Final Technical Report

Bacterial Degradation of DNT and TNT Mixtures
CU1212

Performing organizations:

Rebecca E. Parales, (lead PI)
The University of California, Davis, CA
(Formerly at the University of Iowa)

Jim C. Spain (Co-PI)
Georgia Institute of Technology, Atlanta, GA
(Formerly at Air Force Research Laboratories, Tyndall AFB, Florida)

Glenn R. Johnson
Air Force Research Laboratories, Tyndall AFB, Florida

October 31, 2005
Version 1

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31 OCT 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Bacterial Degradation of DNT and TNT Mixtures	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Parales, Rebecca Spain, Jim Johnson, Glenn R.	**CU 1212**
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of California Section of Microbiology 266 Briggs Hall Davis, CA 95616 Georgia Institute of Technology, Atlanta, GA Air Force Research Laboratories, Tyndall AFB, Florida	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
Strategic Environmental Research & Development Program 901 N Stuart Street, Suite 303 Arlington, VA 22203	**SERDP**
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

The original document contains color images.

14. ABSTRACT

The major objective of this research project was to develop bacterial strains with the ability to efficiently degrade mixtures of dinitrotoluene (DNT) isomers and expand that degradation capability to include TNT. Since most contaminated sites contain mixtures of nitroarene compounds, these strains would have the potential for use in the bioremediation of field sites. We have isolated strains that can degrade several nitroarene compounds, including nitrobenzene, 2-nitrotoluene (2NT) and 2,4- and 2,6-DNT. A clear understanding of the biochemistry, physiology, and molecular genetics of these pathways is a necessary prerequisite for pathway engineering. To this end, we characterized the pathways and enzymes involved, investigated regulation systems, and identified the inducing molecules.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**99**	

Table of Contents

List of Acronyms

NBDO, nitrobenzene dioxygenase; 2NTDO, 2-nitrotoluene dioxygenase; DNTDO, dinitrotoluene dioxygenase; NB, nitrobenzene, NT, nitrotoluene; DNT, dinitrotoluene; ADNT, aminodinitrotoluene; DANT, diaminonitrotoluene; HADNT, hydroxylaminodinitrotoluene; TNT, trinitrotoluene; TAT, triaminotoluene; GC-MS, Gas chromatography, mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

List of Figures

.

List of Tables

Acknowledgments

We gratefully acknowledge the scientific contributions of Shirley Nishino, Dr. Matthew Eby, Dr. George Paoli, Dr. Kyung-Seon Lee, Dr. Kwang-Pil Choi, Juan Parales, Dr. Daniel Lessner, Kou-San Ju, Dr. Rosmarie Friemann, and Dr. S. Ramaswamy. We also thank Dr. David Gibson for helpful suggestions, and the use of his research laboratory and equipment.

Executive Summary

Many sites at DoD installations are contaminated with explosives and related compounds as a result of the manufacture, handing and storage of munitions. There are 25 major Army sites that have been involved with explosives production and the costs for cleanup are enormous. Contamination of firing ranges is a major concern that can interfere with training and readiness. TNT has been the main explosive used by the US military and along with dinitrotoluenes is the major contaminant at the sites. A considerable amount of work has been done to excavate and treat the most heavily contaminated soil at most of the sites. The most common technologies involve incineration or composting, which are very expensive. Bioremediation of TNT has been limited to composting, slurry phase anaerobic reduction, and combinations of bioremediation and iron that are expensive and do not lead to mineralization. The majority of the less heavily contaminated soil and groundwater have not been treated because there is no cost effective strategy for destruction of TNT in water and in soil that has not been excavated. The hazards that DNT, TNT, and their transformation products pose has led to efforts to understand the fate of the compounds in the environment. Research focused on the microbial transformation and bioremediation of nitroaromatic compounds has generally involved cometabolism by microorganisms that do not use the compounds as a growth source. Recent evidence indicates that the presence of nitroaromatic compounds in the environment has a led to the evolution of microorganisms able to degrade many nitroaromatic compounds, but DNT and TNT contamination in the environment continues to be a long-term problem.

Bacterial strains capable of mineralizing nitrobenzene, 2-nitrotoluene (2NT), 2,4-DNT, and 2,6-DNT can be used for bioremediation in self-sustaining, controlled processes. The degradation pathways in such strains have been determined and some of the genes encoding key enzymatic steps have been cloned and sequenced. However, degradation is typically slow and nothing is known about the degradation of mixtures of compounds that would be encountered in the environment. Very little is known about the regulation of the genes encoding the enzymes for nitroarene degradation. Neither 2,4-DNT- nor 2,6-DNT-degrading strains can attack TNT. The goal of this project was to develop a strategy and biocatalyst for the aerobic degradation of TNT supported by DNT as the electron donor. It is clear from studies of DNT degradation that successful engineering of a pathway for the degradation of TNT requires the development of a strategy to introduce oxygen into the ring of TNT. The second requirement is the modification of the system for regulation of the nitroarene dioxygenase genes so that TNT is recognized as an inducer. The final requirement for a productive TNT pathway is the development of a strategy for the metabolism of the intermediates produced by the initial oxidation. The results of the project demonstrate significant progress towards this goal. We have characterized the genes and key enzymes required for nitrobenzene, 2,4-DNT, and 2,6-DNT degradation and found that the origin of the nitroarene degradation pathways traces back to an ancestral naphthalene degradation pathway. The substrate specificities of several nitroarene dioxygenases were characterized, and mutant forms of the enzymes with altered specificities were generated. The results indicated that several nitroarene dioxygenases have the ability to remove nitro groups from aminodinitrotoluenes (ADNTs), which result from nonspecific reduction of TNT. Based on this information, a strategy for TNT degradation that involves an initial reduction to ADNT was developed. The initial reduction is followed by oxidation to an aminonitrocatechol, and finally by ring cleavage. The discovery of enzymes that catalyze all of the steps in the conversion of

TNT to ring fission products containing no nitro groups provides the basis for the construction of a degradation pathway by metabolic engineering. Characterization of the regulation of nitroarene dioxygenase genes in several strains of bacteria revealed a potential regulatory gene for use in the constructed TNT degradation pathway. The encoded protein responds to mononitrotoluenes, DNTs, ADNTs, and TNT as inducers and therefore has the potential to allow expression of the constructed pathway in the presence of the contaminants to be destroyed. Several bacterial strains that were examined have the potential to host the recombinant pathway. A first-generation engineered *Pseudomonas fluorescens* strain in which the multiple operons required for 2,4-DNT degradation were combined as a single unit under the control of a single regulator was capable of growth on 2,4-DNT. The engineered pathway requires optimization, but this achievement paves the way for further metabolic engineering to develop a strain that efficiently degrades both DNT and TNT.

Although an optimized recombinant strain capable of DNT and TNT degradation is not yet available, all of the essential components have now been identified from various nitroarene-degrading bacterial strains. Our results allow us to envision an ideal biocatalyst and the necessary steps to develop such a microbe. The organism would contain a nitroreductase that would specifically attack the nitro group of TNT at the 2-carbon position to produce 2ADNT. To further optimize the pathway, it may be necessary to identify and overexpress a nitroreductase specific for the 2-carbon position of TNT in order to improve the rate of conversion of TNT to 2ADNT. *Burkholderia cepacia* JS872, or another native DNT-degrading strain in our collection, is a candidate host for the final engineered pathway. The attractiveness of strain JS872 lies in the fact that it contains a nitroreductase that is specific for the nitro group at the 2-carbon position. Strain JS872 is also one of the most efficient 2,4-DNT degrading strains known. The regulator of the 2NT degradation pathway is a good candidate to regulate the expression of the proposed pathway in response to nitroarene inducers. NtdR causes a high basal level of expression even in the absence of inducers, but the genes under its control are expressed at even higher levels when mononitrotoluenes, DNTs, ADNTs, or TNT are present. The 2,4-DNT degradation pathway is organized in multiple operons in the three strains that have been analyzed in detail. We do not yet know how the operons encoding the lower pathways are regulated, but we can bypass that difficulty by utilizing the newly constructed single hybrid DNT degradation operon and placing it under the control of NtdR. The fate of ring fission products from TNT will be determined, and the engineered organism will be tested for the ability to grow on TNT. If necessary, the engineered organism will be subjected several to rounds of directed evolution to improve TNT degradation. Finally, the optimized organism will be tested for the ability to degrade DNT and TNT mixtures in microcosms or bioreactors.

Once optimized, the engineered organism would be capable of mineralizing TNT as well as DNT and would be free of the constraints now associated with cometabolic strategies for TNT degradation. It could be used for ground water remediation as well as soil applications. The lack of a requirement for primary growth substrates other than the contaminants of concern means that it could be used to effectively seed firing ranges and remote contaminated sites without the addition of massive amounts of other compostable materials, thus avoiding both materials handling and storage costs as well as disruption of the primary use of the site.

Objectives and Approach

The major objective of this research project was to develop bacterial strains with the ability to efficiently degrade mixtures of dinitrotoluene (DNT) isomers and expand that degradation capability to include TNT. Since most contaminated sites contain mixtures of nitroarene compounds, these strains would have the potential for use in the bioremediation of field sites. We have isolated strains that can degrade several nitroarene compounds, including nitrobenzene, 2-nitrotoluene (2NT) and 2,4- and 2,6-DNT. A clear understanding of the biochemistry, physiology, and molecular genetics of these pathways is a necessary prerequisite for pathway engineering. To this end, we characterized the pathways and enzymes involved, investigated regulation systems, and identified the inducing molecules. Our original proposal included the development of dioxygenase enzymes that oxidize TNT (go/no go). Early in the project, it appeared that this was not feasible and an alternative strategy involving an initial reduction of TNT was adopted, because we had in hand dioxygenases that were capable of oxidizing aminodinitrotoluenes (ADNTs). The key to the proposed work is the use of DNT degradation to support the destruction of TNT. This strategy takes advantage of the fact that TNT and DNT are commonly found as co-contaminants and that DNT supports both the growth of bacteria and the induction of the appropriate degradative enzymes.

Disposal practices associated with TNT manufacturing have resulted in contamination problems at ammunition production and handling facilities worldwide. Soil and groundwater at such sites are extensively contaminated with mixtures of TNT and DNT in varying proportions. Conventional cleanup for such sites includes incineration and variations on composting (5), and both technologies are expensive. Costs for incineration include financial costs, public opposition to locally sited incinerators, problems associated with transport of contaminated materials offsite, and eventual disposal of the incinerated soil. Composting and related technologies for biological destruction of explosives are basically cometabolic processes that require inputs of organic materials to support the destruction of the contaminants of concern. Composting is only applicable to excavated soil because of the requirement for added growth substrates and intensive mixing and handling not only to sustain the composting activities but to control the process so that toxic end products do not accumulate. In contrast to cometabolic strategies (transformation of compounds that do not directly serve as growth substrates for microorganisms), strategies that rely on mineralization target activities that are of direct benefit to microorganisms, that is, mineralizable compounds serve as growth substrates. Moreover, the end products of mineralization are carbon dioxide, water, small inorganic ions, and cell mass. Sustainable strategies that rely on mineralization might reduce costs dramatically.

To date, no organisms have been discovered that can reliably and extensively mineralize TNT at realistic field concentrations. In contrast, nitrobenzene, 2-nitrotoluene (2NT), 2,4-DNT and 2,6-DNT can be mineralized by bacteria in self-sustaining, controlled processes. We determined the degradation pathways and cloned and sequenced the genes encoding the key enzymatic steps (1-4, 6-12, 14-16). In each pathway, degradation is initiated by a multicomponent dioxygenase system that is very similar to the well-studied naphthalene dioxygenase system. Each enzyme consists of an iron-sulfur flavoprotein reductase, and an iron-sulfur ferredoxin that transfer electrons from NAD(P)H to the catalytic oxygenase component.

Each oxygenase consists of large (□) and small (□) subunits. The reaction products are catechols, which are further degraded by *meta* ring-fission pathways (Figure O-1).

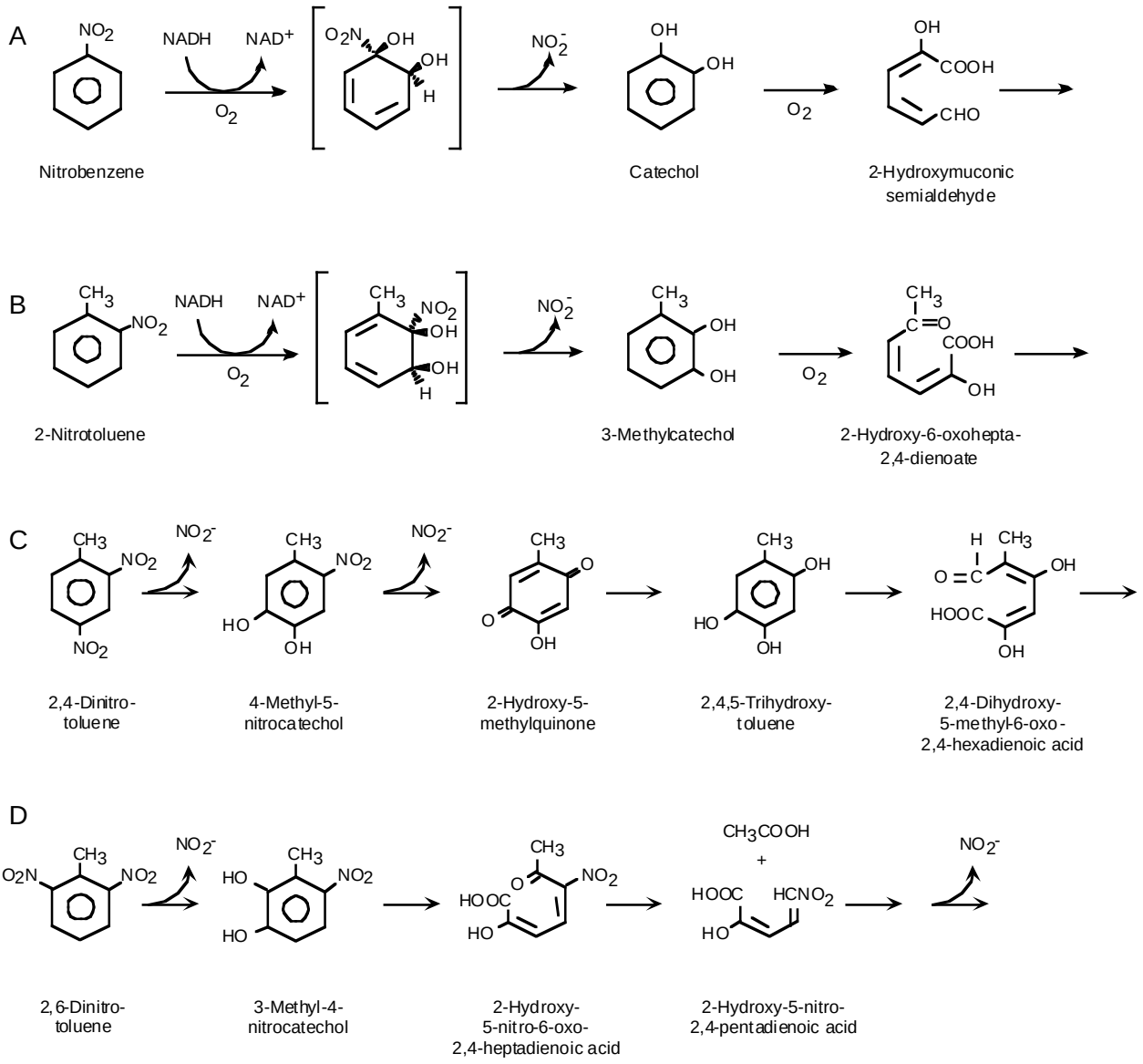


Figure O-1. Pathways that lead to mineralization of nitroaromatic compounds.

The major bottleneck to mineralization of TNT has been the lack of biochemical strategies for initial enzymatic attack on the molecule. There is some information on the enzymes that reduce the nitro groups, but the reactions lead only to transformation and binding of the molecule. In contrast, bacteria able to degrade dinitrotoluenes can oxidatively remove the nitro groups of DNT but do not attack TNT at significant rates. To date, the failure to develop an effective biochemical strategy for oxidative TNT degradation is due to a lack of fundamental

understanding of the degradation of mixtures of the DNT isomers and of induction of the pathways involved.

At the start of this project we had recently discovered bacteria that grow on both isomers of DNT, which meant that they were able to remove nitro groups in both the 2- and 4- positions, and might, therefore, be able to remove similar nitro groups from TNT. The original strategy was to determine whether such enzymes could attack the nitro groups of TNT at detectable rates, then to use metabolic engineering to improve on the natural capabilities. If any one nitro group could be removed, rather than reduced, the resulting molecule might be a mineralizable growth substrate for existing DNT-degrading bacteria. An alternative strategy would be to take advantage of ubiquitous, non-specific nitroreductases that readily reduce one or more of the nitro groups of TNT under aerobic conditions. The resulting aminodinitrotoluenes might then serve as cometabolic substrates for enzymes of the DNT-degradation pathways. What distinguishes this cometabolic strategy from all others designed to bioremediate TNT is that DNT already exists as a co-contaminant at the sites most heavily contaminated with TNT. DNT is a byproduct of TNT manufacture and earlier work demonstrated that DNT degradation can be self-sustaining at contaminated field sites with appropriate controls on pH and O_2 (13). The degradation of mixtures of TNT and DNT would eliminate the requirement for excavation and materials handling. The first requirement for this strategy to be successful is to demonstrate that the enzymes that catalyze the reactions in the DNT degradation pathways can transform the corresponding TNT metabolites.

Regardless of the strategy for engineering a TNT-degrading organism, basic principles apply. First, one must find enzymes that have some native ability to catalyze the reaction of interest. Then, one must generate diversity in order to be able to select for improved variants. The next task is to recruit and assemble genes to encode complete pathways, and finally regulation and flux must be optimized. The proposal to use DNT-degrading bacteria as the platform for metabolic engineering was in the expectation that the native ability to catalyze a number of necessary reactions for TNT degradation would already be present in the enzymes of the DNT pathway. For the first strategy to work, DNT-dioxygenases would have to be able to remove nitro groups from the TNT molecule. Directed evolution techniques would then be applied to generate dioxygenase variants with greater affinities for TNT. The result of such an attack on TNT would be a methyldinitrocatechol. The next candidate enzyme would be tested for the ability to either catalyze the removal of a second nitro group, or to effect ring fission of the methyldinitrocatechol, and then the genes would subjected to a round of directed evolution to optimize that step. Each step in the native pathway could be altered in turn until a complete pathway is realized.

The alternate strategy, illustrated below (Figure O-2), was applied when it became apparent that the extant nitroarene dioxygenases do not attack TNT, but that they are able to transform ADNTs. TNT is readily transformed to ADNTs by a wide range of organisms. Therefore, the subsequent efforts focused on determining whether the amino derivatives were transformed by the appropriate enzymes of the DNT-degradation pathways, and to improve the activity by directed evolution, and/or to develop strategies to avoid misrouting of the transformation products.

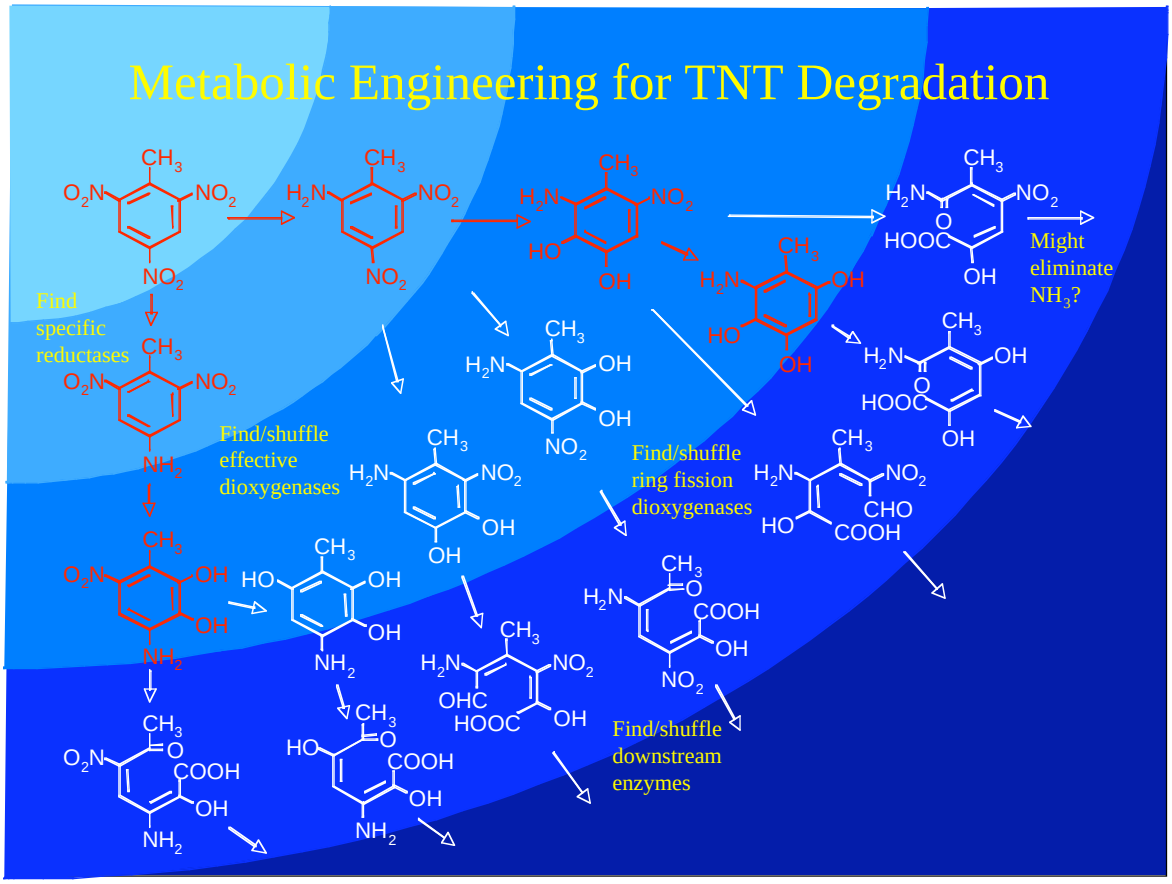


Figure O-2. Strategy for the development of a metabolic pathway for TNT degradation. Enzymes are known for steps indicated in red.

Bottlenecks to the assembly of a TNT degradation pathway included enzyme specificity, lack of a nitroreductase, regulation, and the reactivity of pathway intermediates that could lead to misrouting. The initial approach for overcoming the bottlenecks was to increase our fundamental understanding of the microbial processes involved in the degradation of DNT. The insight about the enzymes involved and their regulation was necessary to develop improved strategies for the degradation of mixtures of the contaminants. The understanding about the regulation and biochemistry of the initial reactions in turn will provide the basis for metabolic engineering to develop a new biochemical strategy for degradation of TNT.

Literature Cited

1. **An, D., D. T. Gibson, and J. C. Spain.** 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. J. Bacteriol. **176:**7462-7467.

2. **Haigler, B. E., S. F. Nishino, and J. C. Spain.** 1994. Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. J. Bacteriol. **176:**3433-3437.
3. **Haigler, B. E., W.-C. Suen, and J. C. Spain.** 1996. Purification and sequence analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp. strain DNT. J. Bacteriol. **178:**6019-6024.
4. **Haigler, B. E., G. R. Johnson, W.-C. Suen, and J. C. Spain.** 1999. Biochemical and genetic evidence for *meta*-ring cleavage of 2,4,5-trihydroxytoluene in *Burkholderia* sp. strain DNT. J. Bacteriol. **181:**965-972.
5. **Jerger, D. E., and P. Woodhull.** 2000. Applications and costs for biological treatment of explosive-contaminated soils in the United States, p. 395-423. *In* Spain, J. C., J. B. Hughes, and H.-J. Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton.
6. **Nishino, S. F., and J. C. Spain.** 1995. Oxidative pathway for the biodegradation of nitrobenzene by *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. **61:**2308-2313.
7. **Nishino, S. F., J. C. Spain, H. Lenke, and H.-J. Knackmuss.** 1999. Mineralization of 2,4- and 2,6-dinitrotoluene in soil slurries. Environ. Sci. Technol. **33:**1060-1064.
8. **Nishino, S. F., G. Paoli, and J. C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66:**2139-2147.
9. **Parales, J. V., A. Kumar, R. E. Parales, and D. T. Gibson.** 1996. Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. Gene **181:**57-61.
10. **Parales, J. V., R. E. Parales, S. M. Resnick, and D. T. Gibson.** 1998. Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the C-terminal region of the a subunit of the oxygenase component. J. Bacteriol. **180:**1194–1199.
11. **Parales, R. E., M. D. Emig, N. A. Lynch, and D. T. Gibson.** 1998. Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems. J. Bacteriol. **180:**2337-2344.
12. **Parales, R. E.** 2000. Molecular biology of nitroarene degradation, p. 63-89. *In* Spain, J. C., J. B. Hughes, and H.-J. Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton.
13. **Spain, J. C., S. F. Nishino, M. R. Green, J. E. Forbert, N. A. Nogalski, R. Unterman, W. M. Riznychok, S. E. Thompson, P. M. Sleeper, and M. A. Boxwell.** 1999. Field demonstration of FBR for treatment of nitrotoluenes in groundwater, p. 7-14. *In* Alleman, B. C. and A. Leeson (ed.), Bioremediation of nitroaromatic and haloaromatic compounds. Battelle Press, Columbus, OH.
14. **Spanggord, R. J., J. C. Spain, S. F. Nishino, and K. E. Mortelmans.** 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **57:**3200-3205.
15. **Suen, W.-C., and J. C. Spain.** 1993. Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation. J. Bacteriol. **175:**1831-1837.
16. **Suen, W.-C., B. E. Haigler, and J. C. Spain.** 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J. Bacteriol. **178:**4926-4934.

Results and Accomplishments

I. DNT degrading strains: isolation and pathway characterization

A variety of DNT-contaminated sites were examined for bacteria that grow on 2,4-DNT and/or 2,6-DNT as the sole carbon, nitrogen and energy source. A number of cultures that degraded mixtures of 2,4-DNT and 2,6-DNT served as the source of new DNT-degrading strains. Isolates were obtained from soil from the Deterrent Burning Ground at Badger Army Ammunition Plant (BAAP), from DNT plumes in ground water at Volunteer Army Ammunition Plant (VAAP), and from soil and ground water at commercial sites in Texas, Florida, and Canada (Table I-1). Strains were isolated from actively degrading cultures as previously described (4).

All of the strains from BAAP were identified by Biolog GN plates as *Achromobacter xylosoxydans* (formerly *Alcaligenes).* Some of the strains grew on 2,4-DNT, others on 2,6-DNT and a small fraction of the isolates were dual degraders and utilized both 2,4- and 2,6-DNT. The strains had a growth optimum at pH 8.3, which is identical to the soil pH, and about 1 pH unit higher than the pH optimum of strains previously isolated from VAAP and a commercial site in West Virginia.

Other isolates from a pilot scale soil slurry bioreactor that degraded DNT in field contaminated soils from BAAP and then Volunteer Army Ammunition Plant (VAAP) were also predominantly *A. xylosoxydans.* Other DNT degrading isolates from Texas, Florida and Canada could not be identified by the Biolog system. Selected strains were identified by 16S rRNA at a commercial lab (Midi Labs, Newark, DE).

The cultures released stoichiometric amounts of nitrite from DNT, and therefore were presumed to utilize the degradation pathways previously established for 2,4-DNT (1, 5) and 2,6-DNT (4). The pathways were confirmed by assays for key enzyme activities in crude extracts of cells grown on either 2,4-DNT or 2,6-DNT. 4-Methyl-5-nitrocatechol monooxygenase was assayed for the 2,4-DNT degradation pathway, and 3-methyl-4-nitrocatechol 2,3-dioxygenase for the 2,6-DNT degradation pathway. Extracts from succinate-grown cultures lacked both activities, which indicated that the DNT-degradation pathways are not constitutive.

Previous studies showed that an *E. coli* bioreporter carrying a *dntR-lux* fusion (2) bioluminesced in the presence of salicylate. That observation coupled with the close relatedness of the *dnt* operon with the *nag* operon (3) suggested that salicylate might stimulate induction of the DNT degradation pathways. Several DNT-degrading strains were presented with a range of concentrations of 2,4-DNT or 2,6-DNT in the presence of a range of concentrations of salicylate. Nitrite release from DNT was assayed after 24 and 72 h. Slightly elevated levels of nitrite were detected in cultures of *Achromobacter xylosoxidans* JS888 when 100 µM or less of salicylate was present in the culture medium. Neither *Burkholderia cepacia* JS872 nor *B. cepacia* R34, both 2,4-DNT degrading strains, showed any elevated nitrite release under the same conditions (Figure I-1).

Table I-1. Identification of DNT-degrading isolates.

Isolate	Source	2,4-DNT	2,6-DNT
Achromobacter xylosoxidans JS886	BAAP	X	X
Achromobacter xylosoxidans JS887	BAAP	X	X
Achromobacter xylosoxidans JS888	BAAP	X	X
Achromobacter xylosoxidans JS889	BAAP	X	X
Achromobacter xylosoxidans JS890	BAAP		X
Achromobacter xylosoxidans JS891	BAAP		X
Pseudomonas carboxydohydrogena JS892	BAAP		X
Aeromonas hydrophila hydrophila JS894	FL		X
Acidovorax sp. JS895	TX	X	
Methylorhabdus sp. JS896	TX	X	X
Achromobacter xylosoxidans JS897	BAAP	X	
Mycobacterium gilyum JS898	TX		X
Nocardiodes sp. JS899	BAAP		X
Variovorax paradoxus JS151	VAAP	X	X
Pseudomonas nitroreducens JS152	VAAP	X	X
Agrobacterium tumefaciens JS153	VAAP	X	X
Burkholderia sp. JS 158	VAAP	X	
Achromobacter xylosoxidans JS180	BAAP		X

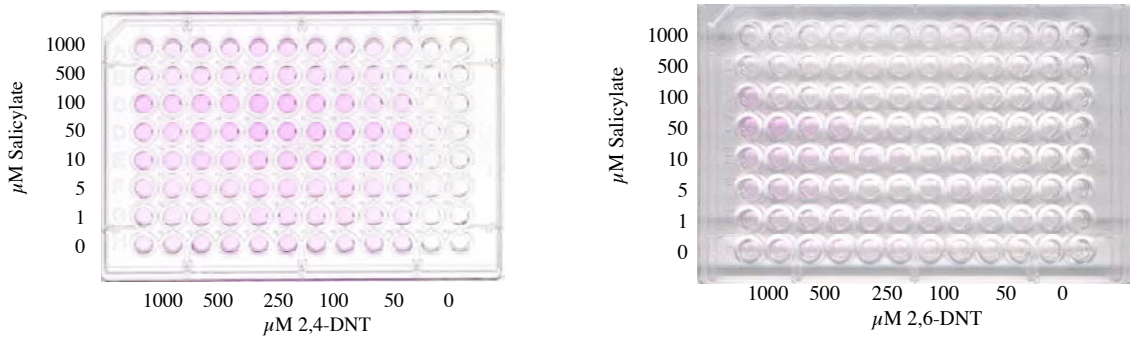


Figure I-1. The dual degrading strain *Achromobacter xylosoxidans* JS888 showed elevated levels of nitrite release from DNT in the presence of 100 µM or less salicylate. 2,4-DNT shown at 24 h, 2,6-DNT at 72 h.

Literature Cited

1. **Haigler, B. E., S. F. Nishino, and J. C. Spain.** 1994. Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. J. Bacteriol. **176:**3433-3437.
2. **Hay, A. G., J. F. Rice, B. M. Applegate, N. G. Bright, and G. S. Sayler.** 2000. A bioluminescent whole-cell reporter for detection of 2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenol in soil. Appl. Environ. Microbiol. **66:**4589-4594.
3. **Johnson, G. R., R. K. Jain, and J. C. Spain.** 2002. Origins of the 2,4-dinitrotoluene pathway. J. Bacteriol. **184:**4219-4232.
4. **Nishino, S. F., G. Paoli, and J. C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66:**2139-2147.
5. **Spanggord, R. J., J. C. Spain, S. F. Nishino, and K. E. Mortelmans.** 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **57:**3200-3205.

II. Characterization of the lower pathway for degradation of 2,4-dinitrotoluene from *Burkholderia cepacia* R34

Background

Development of a sustainable strategy for degradation of TNT and its congeners will require that nitroarenes be catabolized to compounds that can enter central metabolic pathways of microorganisms. An understanding of the enzymes that allow transformation of intermediates in nitroarene degradation to typical metabolic intermediates is critical to predicting and controlling the strategy. Previous work in our laboratory defined the pathway and characterized enzymes for catabolism of 2,4-DNT through ring fission (2). During our funding under SERDP project CU-1212, we extended the understanding of the lower pathway for 2,4-DNT degradation by identifying catabolic steps for transformation of the ring-fission product to ultimately yield pyruvate and propionyl-CoA. The work was included in manuscript on evolution and characterization of the 2, 4-DNT pathway from *Burkholderia cepacia* R34 (3).

Results

The region encoding the 2,4-DNT lower pathway was localized from activity provided from recombinant strains carrying subcloned DNA fragments of the R34 genome. Subsequent analysis of nucleotide sequence data identified specific open reading frames and allowed further dissection of the pathway through isolation or disruption of individual genes. Cell lysates derived from recombinant *E. coli* strains and strain R34 were used to study degradative steps following ring fission in the 2,4-DNT pathway. The 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoate (DMOH) isomerase / 4-hydroxy-2-keto-5-methyl-6-oxo-3-hexenoate (HKMOH) hydrolase activities were evaluated qualitatively using spectrophotometry, scanning the UV-vis absorbance of reaction mixtures from 220-425 nm. Quantitative assays of DMOH isomerase / HKMOH hydrolase activity measured time dependent accumulation of products using HPLC. The substrate (DMOH) was produced enzymatically from 2,4,5-trihydroxytoluene (THT) using excess THT oxygenase (DntD) supplied in cell extracts of *E. coli* (pJS333) (4).

The genes encoding the 2,4-DNT lower pathway are clustered in a relatively compact area. The coding region was identified by ability to confer growth on 2,4,5-trihydroxytoluene to *E. coli* host strains. The growth indicated that the DNA fragment cloned in pJS336 includes genes for enzymes that catalyze transformation of the ring-fission product to compounds for intermediary metabolism in *E. coli*. Analysis of the nucleotide sequence in the region revealed six open reading frames, *dntD* (encoding the ring fission enzyme (4)) and five downstream ORFs that were designated *dntE*, ORF13, *dntG*, ORF9, and ORF10 (Figure II-1; Table II-1). Comparisons of the deduced amino acid sequences from the gene products to previously characterized enzymes provided insight to potential roles of the enzymes in the pathway. Subsequent enzyme assays characterized the steps following ring fission that are catalyzed by the *dntG*-gene product.

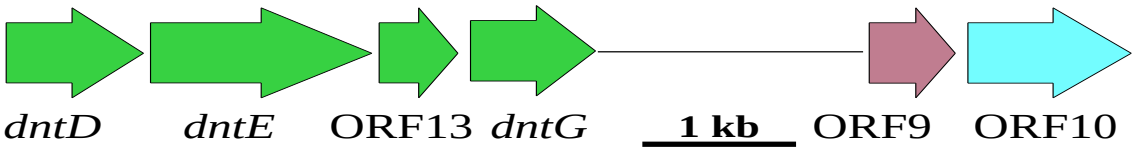


Figure II-1. Organization of the 2,4-DNT lower-pathway genes. Reverse transcriptase-PCR assays showed that the *dntD-dntG* region is operonic, yielding a single mRNA molecule, ORF9 and 10 appear to be transcribed separately from *dntD-dntG*.

Table II-1. The 2,4-DNT lower pathway gene cluster. Description of the gene products and their roles in pathway are based on activities of similar proteins and results from enzyme assays.

Gene Product	**Description**	**Role in 24DNT degradation ?**
DntD	2,4,5-THT oxygenase -Extradiol ring-fission enzyme	Yes
DntE	Methylmuconate semialdehyde dehydrogenase	Likely
ORF13	Similar to NADH-dependent reductase	Unknown
DntG	2,4-Dihydroxy-5-methyl-6-oxo-2,4-hexadienoate (DMOH) isomerase / 4-hydroxy-2-keto-5-methyl-6-oxo-3-hexenoate (HKMOH) hydrolase	Yes
ORF9	Similar to NADH-dependent reductase	Unknown
ORF10	Similar to maleylacetate reductase	Unknown

Analysis of the nucleotide and deduced amino acid sequence of the lower pathway genes (Fig II-1) provided insight to the degradative pathway and its evolutionary development. The *dntD* gene product and its ring fission reaction was previously characterized (4). The *dntE* gene product was similar to the CoA-dependent methylmalonate semialdehyde dehydrogenases used for the valine degradation pathway in microbes and mammals. The deduced amino acid sequence of DntG shared significant identity with cycloisomerases (tautomerases) from various lower pathways and fumarylacetoacetate (FAA) hydrolases from the phenylalanine/tyrosine degradation pathway. Alignments of the DntG amino acid sequence with its homologs showed conservation of residues key to catalytic functions. The conservation suggested that DntG could catalyze similar reactions in the 2,4-DNT pathway. Two ORFs in the region, ORF13, and ORF9, are distantly related to putative NADH-dependent reductases. The function of the gene products from R34 and their homologs noted in databases are not well understood. The proposed activity appears to be based upon their sequence similarity to prototypical NADH-dependent reductases. Curiously, ORF13 and ORF9 share greater identity with one another (62%) than any other entries in GenBank database. The identity might reflect a gene duplication event, but significant divergence has occurred since that event. The final gene product in the region, ORF10, encodes a 452-amino acid polypeptide that is similar to several maleylacetate reductases. Maleylacetate reductases are typically found in ortho-ring fission pathways such as those for degradation of chloroaromatic compounds (1). The anomalous association with the meta-ring fission pathway and significant distance away from *dntDEFG* are signs that ORF9 and ORF10 are not associated with the 2,4-DNT pathway and might be artifacts remaining from assembly of the region.

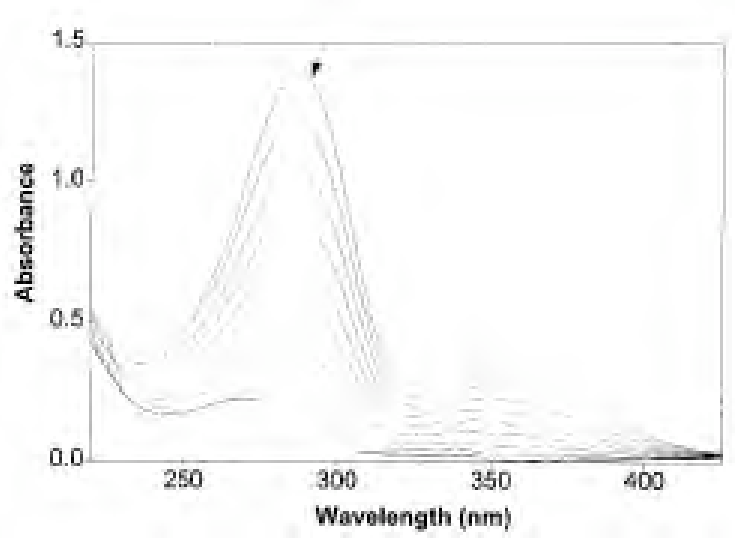


Figure II-2. Enzymatic transformation of 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOH) (from (3)). The arrow indicates first spectral scan of reaction containing the THT-ring fission product, DMOH, and cell lysate from recombinant *E. coli* carrying *dntG*. Spectra were collected every 20 seconds.

The metabolic step following ring fission in the 2,4-DNT pathway had not been defined prior to work summarized here. Results from the enzyme assays indicated that DntG catalyzed the next step in the pathway. Cell extracts from strains that carried *dntG* catalyzed rapid disappearance of the 2,4,5-THT ring fission product (Figure II-2). A series of deletion derivatives and subclones that allowed isolated synthesis of the other gene products shown in Figure II-1, did not provide activity to transform of the ring fission product. The spectral scans during the reaction reveal an initial shift in λ_{max} from 278 nm to 270 nm, then as the reaction progressed, the compound at 270 nm was transformed to UV-transparent compounds (Figure II-2). The stepwise change in the spectrum and absence of an isosbestic point indicated that there is not a direct conversion between DMOH and the final reaction products. The result supports the hypothesis that DntG provides dual catalytic activities (isomerase/hydrolase) like its homolog the HHDD isomerase/OPET decarboxylase from the homoprotocatechuate degradation pathway (5).

The products of the DntG-catalyzed reaction were analyzed using HPLC (Figure II-3). Pyruvate and methylmalonate semialdehyde (2-methyl-3-oxo-propionic acid) (Figure II-3 inset) were proposed products based on analogy to reactions catalyzed by the fumarylacetoacetate hydrolase of the tyrosine pathway and fumarylpyruvate hydrolase from the gentisate degradation pathway. The HPLC analysis revealed two organic products that co-chromatographed with pyruvate and methylmalonate semialdehyde chemical standards. Quantitation of the pyruvate indicated that it was produced in stoichiometric amounts from the ring fission product, the relative rate of methylmalonate semialdehyde accumulation was parallel to the pyruvate formation (Figure II-3). The results are consistent with the proposed hydrolysis reaction.

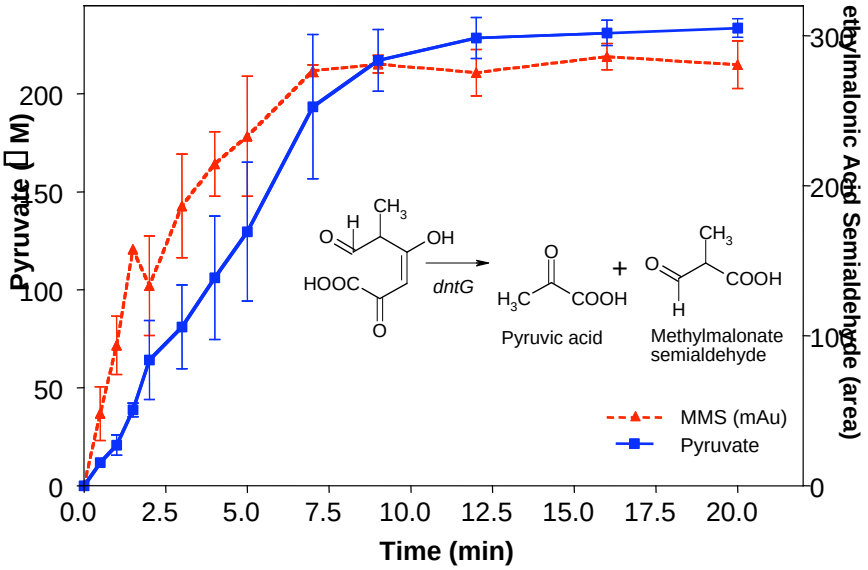


Figure II-3. Conversion of 4-hydroxy-2-keto-5-methyl-6-oxo-3-hexenoate (HKMOH) to pyruvate and methylmalonate semialdehyde.

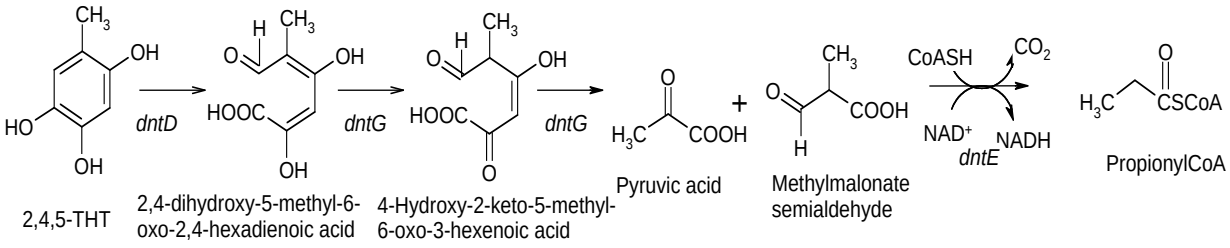


Figure II-4. Lower pathway for 2,4-DNT degradation. The DntE-catalyzed reaction and product are proposed by analogy to previously characterized enzymes and degradative pathways.

Conclusions

The degradative steps in the 2,4-DNT lower pathway were proposed from characterization of reaction products and projected from reactions catalyzed by homologous enzymes (Figure II-4). The methylmalonate semialdehyde that results from hydrolysis of 4-hydroxy-2-keto-5-methyl-6-oxo-3-hexenoic acid (Figure II-4) is identical to the intermediate in the valine degradation pathway. In valine degradation, the next step is the oxidative decarboxylation of the methylmalonate semialdehyde to yield propionyl-CoA. The reaction is catalyzed by methylmalonate semialdehyde dehydrogenase, an apparent homolog of *dntE*. Accordingly, the final step proposed in Figure II-4, seems quite likely, but has not been demonstrated experimentally. The most significant aspect of the work relevant to CU1212 is defining the degradation of the nitroarene (2,4-DNT) to common intermediary metabolites. The understanding can be applied to engineering pathways for TNT or other polynitro-aromatic compounds.

Literature Cited

1. **Gottschalk, G.** 1986. Bacterial metabolism, 2nd ed. Springer-Verlag, New York.
2. **Haigler, B. E., G. R. Johnson, W.-C. Suen, and J. C. Spain.** 1999. Biochemical and genetic evidence for *meta*-ring cleavage of 2,4,5-trihydroxytoluene in *Burkholderia* sp. Strain DNT. J. Bacteriol **181:**965-972.
3. **Johnson, G. R., R. K. Jain, and J. C. Spain.** 2002. Origins of the 2,4-dinitrotoluene pathway. J. Bacteriol. **184:**4219-4232.
4. **Johnson, G. R., R. K. Jain, and J. C. Spain.** 2000. Properties of the trihydroxytoluene oxygenase from *Burkholderia cepacia* R34; an extradiol dioxygenase from the 2,4-dinitrotoluene pathway. Arch. Microbiol. **173:**86-90.
5. **Roper, D. I., and R. A. Cooper.** 1993. Purification, nucleotide sequence and some properties of a bifunctional isomerase/decarboxylase from the homoprotocatechuate degradative pathway of *Escherichia coli* C. Eur. J. Biochem. **217:**575-580.

III. Biodegradation of 2,6-dinitrotoluene by *Achromobacter xylosoxidans* subsp. *xylosoxidans* strain JS180

Summary

We have characterized the catabolic pathway responsible for biodegradation of 2,6-DNT from the environmental isolate *Achromobacter xylosoxidans* strain JS180. These findings show that the 2,4- and 2,6-DNT degradative pathways are distinct, except for the initial step, in which they share a similar enzyme and mechanism. Organization of the catabolic genes in the genome of JS180 and assay of their encoded activities reveal mechanisms of recent and continuing evolution. An understanding of these mechanisms *in situ* is important to bioremediation strategies, particularly natural attenuation.

Included in this study is the purification and characterization of a novel extradiol dioxygenase, which attacks 3-methyl-4-nitrocatechol. This is the first example of a ring fission dioxygenase that catalyzes the oxidation of a nitroarene as its physiological role. Isolation of JS180 from DNT-contaminated soil and identification of the pathway for complete detoxification of 2,6-dinitrotoluene provides evidence that 2,6-DNT is removed from contaminated sites by indigenous microflora. Continuing studies of 2,6-DNT degrading strains will lead to better approaches to site cleanup. Isolation of the enzyme and genes involved in 2,6-DNT degradation can now be used in directed evolution studies to enhance activity on possible metabolites of TNT degradation.

Background

Previous chromatographic and spectroscopic analyses of metabolic intermediates during growth of bacterial strains on 2,6-DNT suggests the preliminary catalytic step of 2,6-DNT degradation is analogous to that for 2,4-DNT [1]. As dihydroxylation of 2,4-DNT by a ring hydroxylating dioxygenase produces 4-methyl-5-nitrocatechol (4M5NC), 3-methyl-4-nitrocatechol (3M4NC) is generated from attack on 2,6-DNT (Figure III-1). 3M4NC was presumed to be the ring-fission substrate to produce a nitro-substituted muconic semialdehyde, which is distinct from the 2,4-DNT pathway. The enzyme responsible for ring fission in the 2,6-DNT degradative pathway is arguably the most important step in understanding how this pollutant is removed in the environment. Our studies were directed on characterizing this step as a focal point in understanding the entire pathway. Also included in this study is preliminary characterization of subsequent degradation steps following ring fission.

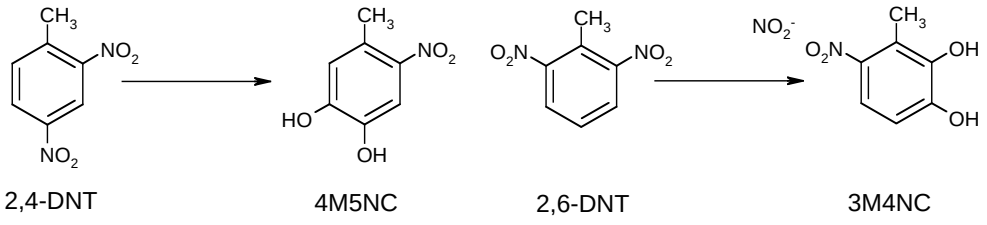


Figure III-1. Comparison of 2,4-DNT and 2,6-DNT dihydroxylation by the DNT dioxygenase.

Results and Discussion

Achromobacter xylosoxidans strain JS180 was isolated from environmental samples of DNT-contaminated soil and groundwater collected from the Badger Army Ammunition Plant (Sauk County, Wisconsin, www.badgeraap.org) using methods described in Nishino et al. [1]. JS180 is able to detoxify 2,6-DNT through the action of four enzyme-catalyzed reactions (Figure III-2).

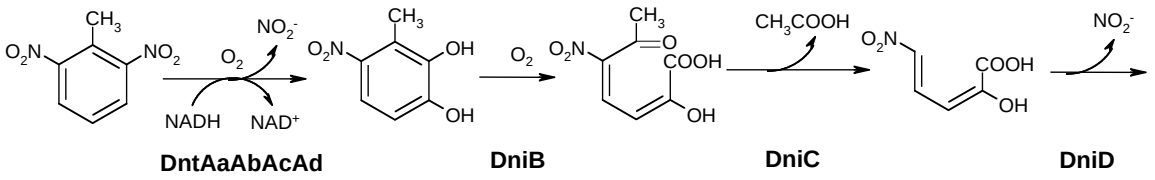


Figure III-2. The 2,6-DNT catabolic pathway from JS180. Enzyme designations are in bold type.

Initial attack of 2,6-DNT and subsequent removal of the first nitro group to form 3M4NC is catalyzed by a multi-component aromatic ring hydroxylating dioxygenase. From the genome of JS180, we have isolated four genes that encode similar activities to those of 2,4-DNT degrading enzymes found in strains DNT and R34. Ring cleavage of 3M4NC is catalyzed by a single component ring fission dioxygenase, DniB, to produce 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid (HNOHA). Purification of the dioxygenase and isolation of the corresponding gene was completed to enable biochemical and genetic characterization. Two separate, purified protein fractions of JS180 cell extract, designated DniC and DniD, catalyzed the conversion of HNOHA to 2-hydroxy-5-nitropenta-2,4-dienoic (HNPA) and removal of the last nitro group from HNPA, respectively.

Genes encoding the aromatic ring hydroylating dioxygenase from JS180

Genomic libraries of JS180 were probed for genes that contained high sequence identity to a conserved DNA region of known nitroarene dioxygenases [2]. Three discrete fragments containing the conserved gene sequence, were isolated from several different genomic libraries. This ensured that a single and unique copy of sequence within the genome matched the DNA probe. When expressed in *E. coli*, the isolated fragment catalyzed the conversion of 2,6-DNT to 3M4NC.

Sequencing of the genomic fragments revealed four genes that encode an aromatic ring hydroxylating dioxygenase and a regulator gene (Figure III-3A). The deduced amino acid sequence of the dioxygenase catalytic subunit is almost identical in sequence to those found in 2,4-DNT degrading strains R34, [2] DNT, [3] and JS872 (unpublished data) (Table III-1). The sequence is also homologous to the catalytic subunit of the naphthalene dioxygenase from *Ralstonia* sp. strain U2 [4]. The genes, which carry the same designation as those encoding the 2,4-DNT dioxygenase (*dntAaAbAcAd)*, were found in the genome of JS180 adjacent to genes encoding reactions for the biotransformation of naphthalene (*nag* genes, Figure III-3B).

Naphthalene, found in low concentration in most natural soils [5], is transformed by bacteria by homologous mechanisms to those involved in the initial attack on dinitrotoluenes [6]. The *nag* genes of JS180 are identical to those found in the naphthalene degrading strain U2 [4]. Insertion elements (IS) directly flank the *dnt* genes in JS180, suggesting that genes were incorporated within the naphthalene catabolic operon by transposition and homologous recombination, which is consistent with mechanisms of chromosomal rearrangements proposed by Gray [7]. IS elements are also found adjacent to the *dnt* genes in the genomes of strain DNT and R34 (not shown) [3, 8].

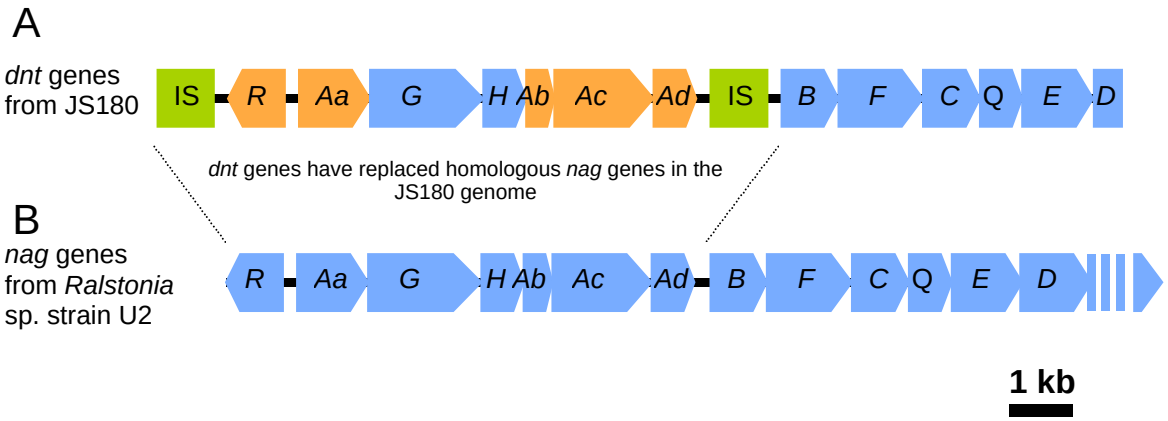


Figure III-3. Organization of the ring hydroxylating dioxygenase genes in the genome of JS180. A sequenced fragment of genomic DNA from JS180 containing the *dnt* gene is shown in A. The *dnt* genes are orange. Genes identical to naphthalene degradation genes (*nag*) are blue and insertional elements are green. Genetic organization of the naphthalene catabolic genes from *Ralstonia* sp. strain U2 is shown in B.

Table. III-1. Amino acid sequence identity matrix between the catalytic subunit of 2,4-DNT and naphthalene dioxygenases. Percent identity of pair-wise alignments is shown for four DntAc protein sequences from the 2,4-DNT degrading strains R34, DNT, JS872, and the 2,6-DNT degrading strain JS180. Also shown are the pairwise alignments of each DntAc sequence to NagAc from the naphthalene degrader *Ralstonia* sp. strain U2.

Strain	**R34**	**DNT**	**JS872**	**U2**	**JS180**
R34	100	93.9	95.7	91.2	95.5
DNT		100	97.0	87.9	96.8
JS872			100	89.2	99.3
U2				100	89.0
JS180					100

When a subclone of the JS180 genome containing the ring-hydroxylating dioxygenase genes was expressed in *E. coli*, the strain transformed 2,6-DNT, 2,4-DNT, and naphthalene (Figure III-4). The relative substrate specificity of the dioxygenase from JS180 is similar to those found in strains DNT and R34, in which the growth substrate 2,4-DNT is preferred over 2,6-DNT [9, 10]. The strain also transformed salicylate, confirming that the two genes in between *dntAa* and *dntAb*, which encode for the salicylate hydroxylase from the naphthalene pathway, are functional (labeled *G* and *H* in Figure III- 3). Although the dioxygenase from JS180 attacks 2,4-DNT and naphthalene, it is unable to grow on these substrates. Salicylate transformation is surprising and contrasts with the situation in all other nitroarene degrading strains studied to date [2, 3, 11, 12]. In other nitrotoluene strains, mutations within the salicylate hydroxylases genes have eliminated function. These genes are not essential, because salicylate is not an intermediate in the 2,4- and 2,6-DNT catabolic pathways. Remnants of the salicylate hydroxylase genes found in nitrotoluene strains suggest that the *nag* genes are the progenitor of *dnt* genes and over time, mutations, which created the structural genes for nitrotoluene degradation, rendered the salicylate genes non-functional [2, 6]. Perhaps the presence of functional salicylate genes in JS180 is indicative of a divergent evolutionary event different and much more recent than the other nitrotoluene degradative pathways.

The ratios at which biotransformation of 2,4-, 2,6-DNT, and naphthalene are degraded by DNT dioxygenases are shown in Table III-2. The substrate specificities of the two dioxygenases from strains R34 and DNT are identical, with very little activity toward 2,6-DNT compared to activity toward 2,4-DNT. In contrast, 2,6-DNT activity by the JS180 dioxygenase is considerably greater in comparison to the activity of dioxygenases from the 2,4-DNT degrading strains, respective to activity on 2,4-DNT. 2,4-DNT as the preferred substrate of the

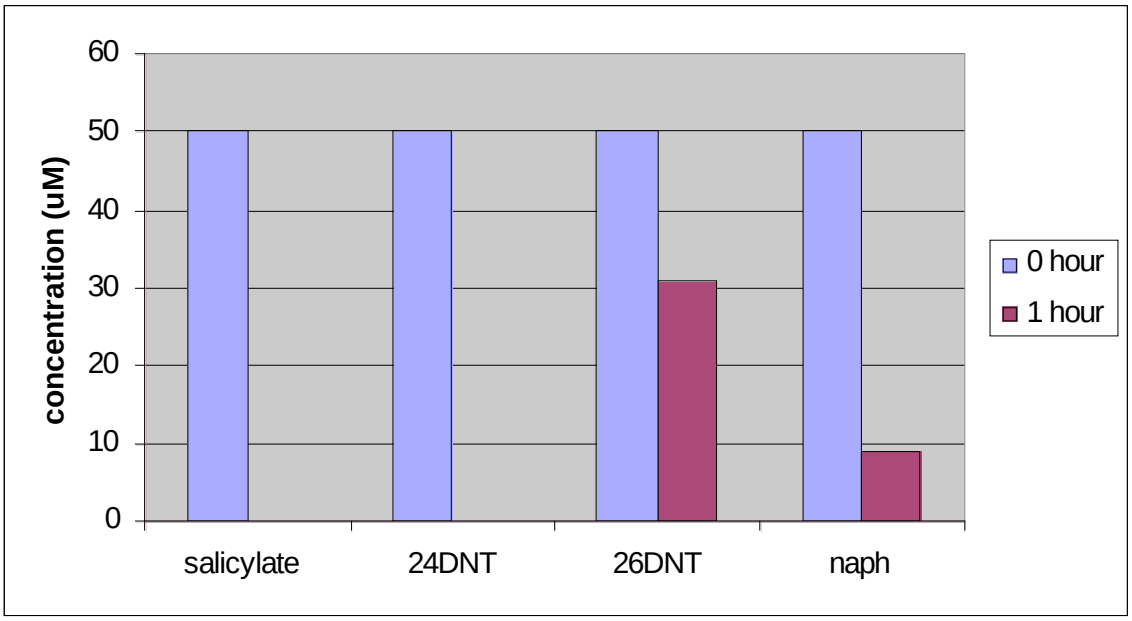


Figure III-4. Transformation of salicylate, 2,4-, 2,6-DNT, and naphthalene by cell suspensions of *E. coli* expressing the ring hydroxylating dioxygenase and salicylate hydroxylase genes from JS180. Cells were grown in a non-specific medium, centrifuged, and resuspended in phosphate buffer containing 50 □M of substrate. Substrate disappearance was monitored by HPLC at the start of the reaction and after one hour.

Table III-2. Comparison of substrate specificity between the DNT dioxygenases. Relative product reaction rates are shown for the substrates 2,4-, 2.6-DNT and naphthalene by recombinant *E. coli* expressing genes encoding the DNT dioxygenases from strains R34, DNT, and JS180. Reaction rates with 2,6-DNT and naphthalene are shown as percentages of the product formation rate from reactions with 2,4-DNT. Strain R34 and DNT reactions were completed by Leungsakul et al. [10] and Suen et al. [3]. Reaction rates from strain JS180 were completed as described in Figure III-4, except reactions contained 100 μM of substrate and product formation was monitored by HPLC over the course of 3 hours.

substrate	product	strain R34	strain DNT	strain JS180
2,4-DNT	4M5NC	100%	100%	100%
2,6-DNT	3M4NC	4%	4%	30%
naphthalene	1,2-dihydroxy-naphthalene	37%	37%	55%

dioxygenase from JS180 is perplexing, considering JS180 does not grow on 2,4-DNT. Under closer inspection, the increased activity toward 2,6-DNT with respect to its 2,4-DNT degrading counterparts does reflect the physiological growth substrate of each strain. We propose that JS180 recruited a dioxygenase initially adapted for 2,4-DNT utilization and that subsequent evolution led to the enhanced affinity for 2,6-DNT.

These findings suggest JS180 has undergone recent evolution from utilization of the more natural carbon source naphthalene to utilizing the man-made pollutant 2,6-DNT. DNT dioxygenase genes are closely related to naphthalene dioxygenase genes, with over 95% of their nucleotide sequence identical. This level of identity could easily facilitate their annealing during replication, which would lead their exchange within genomes. The presence of IS elements flanking *dnt* genes in the genomes of DNT degraders is residual evidence of such an event. The relative broad substrate specificity of the JS180 dioxygenase and the existence of intact *nag* genes and functional salicylate hydroxylase genes in the genome of JS180 support a hypothesis that evolution of the initial steps in 2,6-DNT degradation evolved very recently from a naphthalene degradative pathway.

Characterization of the ring fission dioxygenase DniB

A single-component dioxygenase was purified from JS180 cells grown on 2,6-DNT as the sole carbon, nitrogen, and energy source. Partial amino acid sequence of the dioxygenase, obtained though fragmentation of the protein and sequencing of resulting peptides, was utilized to create degenerate PCR primers. One degenerate primer pair amplified a portion of the gene from boiled cells. The gene fragment was made into a hybridization probe and successfully used to isolate an 8.6 kb fragment of genomic DNA from JS180 containing the dioxygenase gene.

Based upon spectrophotometric evidence, [1] DniB catalyzes cleavage of 3-methyl-4-nitrocatechol to 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid (Figure III-5). DniB is active as a monomer of between 32 kDa and 38 kDa in size as determined by size exclusion chromatography. The deduced amino acid sequence of the isolated *dniB* gene confirms that

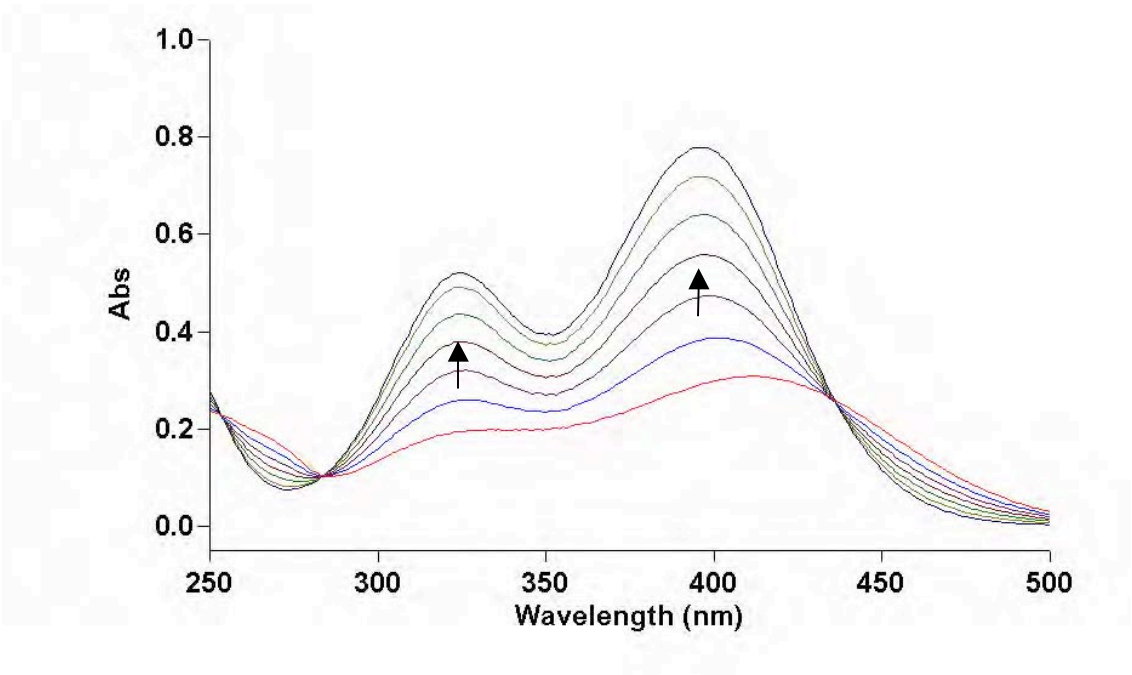


Figure III-5. Conversion of 3M4NC to HNOHA by DniB. Catalytic activity was monitored by UV/VIS spectrometry. The reaction contained 20 M of substrate and 0.2 mg protein in 0.5 ml total volume (50 mM phosphate buffer, pH = 7). The initial absorbance is represented by the red line and progressed in the direction shown by the arrows. Spectra were recorded in 1 min intervals.

DniB belongs to the type I, two domain extradiol dioxygenase family [13]. Other than amino acid residues important for catalytic activity, DniB is distantly related to other dioxygenases in its class (Figure III-6). Closest relatives of DniB are the biphenyl ring fission dioxygenases found in polychlorinated biphenyl and dibenzofuran degrading *Rhodococcus* sp. strains K37, RHA1, and YK2. The amino acid sequence of DniB is approximately 31% identical and 49% similar to these dioxygenases.

Conversion of the ring fission product and removal of the nitro group by DniC and DniD.

Two fractions from anion exchange and size exclusion column chromatography of JS180 total protein catalyzed conversion of HNOHA to HPNA (Figure III-7) and nitrite release from HPNA, respectively. Conversion of HNOHA to HPNA is completed through hydrolytic cleavage of a carbon-carbon bond, which releases acetate from HNOHA [1]. Further purification of active fractions which catalyzed HNOHA to HPNA led to the isolation of a 67 kDa protein, designated DniC. Conversion of HPNA by partially purified protein, designated DniD, catalyzes the removal of the second nitro group from 2,6-DNT without the addition of cofactors, such as electron donor or acceptors. Future studies will be completed to isolate the genes involved and to determine the final product of 2,6-DNT degradation. Removal of the last nitro group is assumed to render the product innocuous.

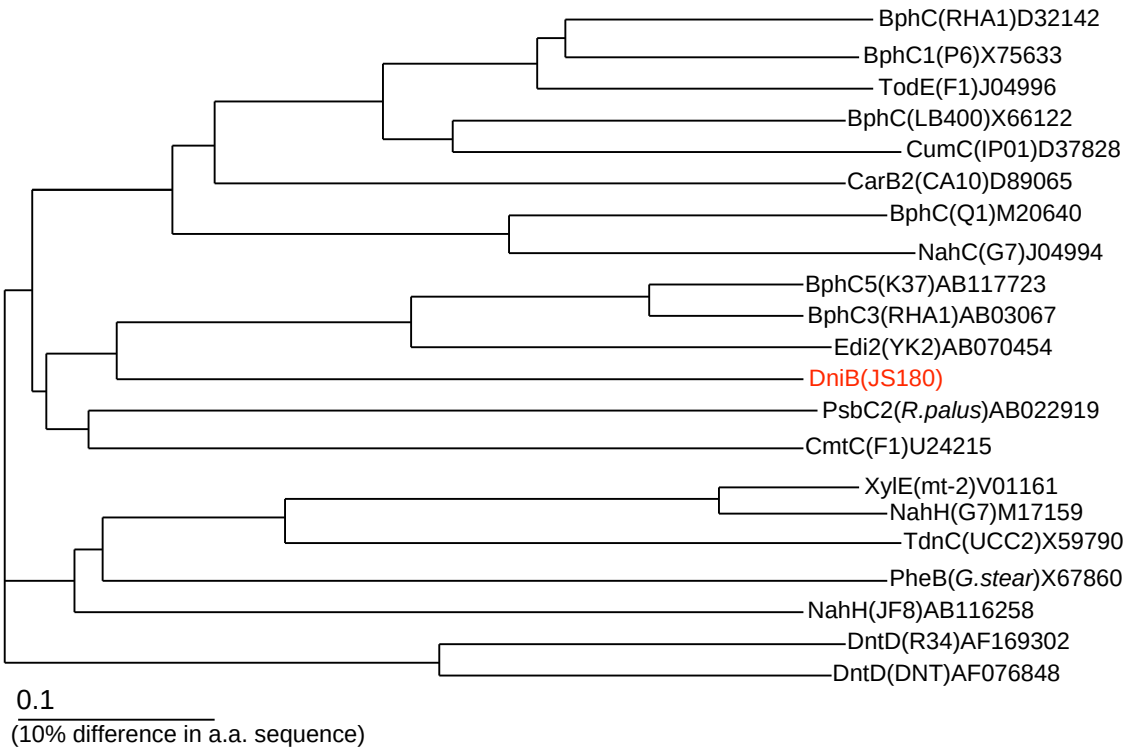


Figure III-6. Extradiol ring fission dioxygenase phylogenetic tree. The protein name, originating strain and GenBank designation are shown for each member. Tree was made based on Clustal X alignments. Detailed information of each protein can be found at http://www.ncbi.nlm.nih.gov.

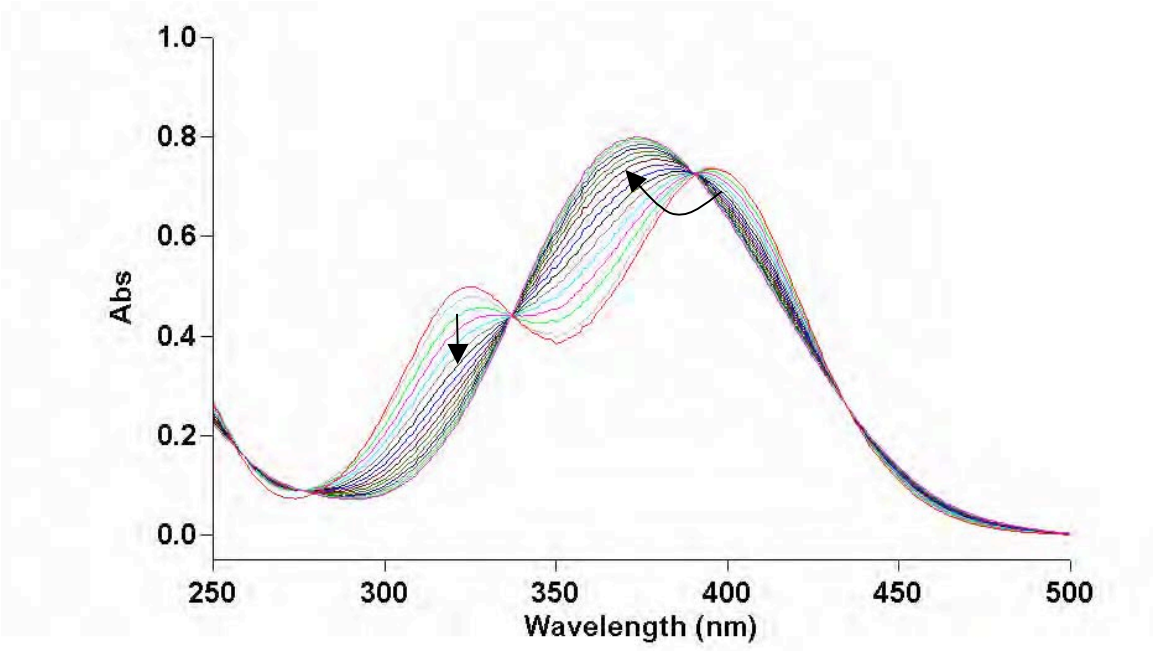


Figure III-7. Conversion of HNOHA to HPNA by DniC. Catalytic activity was monitored by UV/VIS spectrometry. The reactions contained 20 M of substrate and 0.1 mg protein in 0.2 ml total volume (50 mM phosphate buffer, pH = 7). The initial absorbance is represented by the red line and progressed in the direction shown by the arrows. Spectra were recorded in 1 min intervals.

Conclusions

Numerous bacterial strains from a wide variety of genera that are able to degrade either or both 2,4- and 2,6-DNT have been isolated from contaminated sites. Very few of these strains have the ability to grow on 2,6-DNT as a sole carbon, nitrogen, and energy source at a rate that facilitates physiological and genetic studies. Whereas several strains that degrade both isomers were isolated by us, their growth on these substrates was significantly slower than those that grow exclusively on one isomer. Furthermore, their phenotypes are difficult to maintain in the absence of selective pressure from both isomers. Strains that grow adequately on 2,6-DNT for study, are unable to degrade 2,4-DNT. These were chosen to characterize the 2,6-DNT degradative pathway and for continuing studies that address bioremediation of this pollutant and the possibility of exploiting the abilities of 2,6-DNT degrading strains to attack TNT.

In our hands, single DNT degraders have been more efficient than dual degraders at removing each isomer from culture media. Evidence suggests that evolution of the 2,4- and 2,6-DNT pathways has been distinct, possibly occurring in separate bacterial populations. If true, then this could explain why our isolated dual degrading strains are not as efficient in degrading both isomers in comparison to single isomer degrading strains. We conclude that a strategy using consortia of distinct 2,4- and 2,6-DNT degrading bacteria would be better suited than using dual degraders for destruction of DNT in pilot scale microcosms of contaminated soil.

A more in-depth understanding of the mechanisms of genetic transfer at contaminated sites would benefit active bioremediation and natural attenuation strategies. As shown through genetic characterization and substrate specificity measurements of the enzymes involved in DNT degradation, DNT catabolic pathways have recently evolved and are undergoing further refinement. Understanding the rate at which bacteria acquire new capabilities to degrade pollutants through evolution and genetic transfer can give us a better assessment of the fate of these compounds in the environment. Such determinations are important for generating more accurate predictions of the persistence and attenuation of pollutants at contaminated sites.

Literature Cited

1. **Nishino, S.F., G. Paoli, and J.C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66**:2139-2147.
2. **Johnson, G.R., R.K. Jain, and J.C. Spain.** 2002. Origins of the 2,4-dinitrotoluene pathway. J. Bacteriol. **184**:4219-4232.
3. **Suen, W.-C., B.E. Haigler, and J.C. Spain.** 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J. Bacteriol. **178**:4926-4934.
4. **Jones, R.M., B. Britt-Compton, and P.A. Williams.** 2003. The naphthalene catabolic (*nag*) genes of *Ralstonia sp.* strain U2 are an operon that is regulated by NagR, a LysR-type transcriptional regulator. J Bacteriol. **185**:5847-53.
5. **Pellizari, V.H., et al.** 1996. Evaluation of strains isolated by growth on naphthalene and biphenyl for hybridization of genes to dioxygenase probes and polychlorinated biphenyl-degrading ability. Appl. Environ. Microbiol. **62**:2053–2058.

6. **Fuenmayor, S.L., et al.** 1998. A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J. Bacteriol. **180**:2522-2530.
7. **Gray, Y.H.** 2000. It takes two transposons to tango: transposable-element-mediated chromosomal rearrangements. Trends Genet. **16**:461-8.
8. **Johnson, G.R. and J.C. Spain.** 2003. Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. Appl. Environ. Microbiol. **62**:110-123.
9. **Keenan, B.G., et al.** 2004. Saturation mutagenesis of *Burkholderia cepacia* R34 2,4-dinitrotoluene dioxygenase at DntAc valine 350 for synthesizing nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone. Appl. Environ. Microbiol. **70**:3222-31.
10. **Leungsakul, T., et al.** 2005. Saturation mutagenesis of 2,4-DNT dioxygenase of *Burkholderia* sp. strain DNT for enhanced dinitrotoluene degradation. Biotechnol Bioeng.:Epub ahead of print.
11. **Lessner, D.J., et al.** 2002. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. **68**:634-641.
12. **Parales, J.V., et al.** 1996. Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. Gene. **181**:57-61.
13. **Eltis, L.D. and J.T. Bolin.** 1996. Evolutionary relationships among extradiol dioxygenases. J. Bacteriol. **178**:5930-5937.

IV. Molecular characterization, regulation, and substrate specificities of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765 and 2-nitrotoluene dioxygenase from *Acidovorax* sp. strain JS42

Background

Comamonas sp. strain JS765 and *Acidovorax* (formerly *Pseudomonas*) sp. strain JS42 were isolated for their ability to grow on nitrobenzene and 2-nitrotoluene, respectively, as sole sources of carbon, nitrogen and energy (8, 18). In each strain, the degradation of the nitroarene compound is initiated by a multicomponent Rieske non-heme iron dioxygenase. The genes encoding the nitroarene dioxygenase from JS42 have been cloned and sequenced and the substrate range has been tested (19). Preliminary results from our laboratory suggested that *Comamonas* sp. strain JS765 harbored a dioxygenase with a substrate range significantly broader than other nitroarene dioxygenases. Previous studies also suggested that the nitrobenzene dioxygenase (NBDO) genes were inducible and the 2-nitrotoluene dioxygenase (2NTDO) genes were constitutive (8, 18).

The goal of this part of the project was to characterize in detail the initial dioxygenases in these strains, and the regulation of the genes encoding them. This included cloning and sequencing of the dioxygenase genes from JS765, purification and characterization of the NBDO and 2NTDO proteins, determination of substrate specificities of the enzymes, and site-directed mutagenesis to identify enzymes with improved activity or specificity. In addition, mutations in putative regulatory genes were generated to verify their functions, chromosomal *lacZ* fusions were generated in order to identify inducers of dioxygenase gene expression and to optimize dioxygenase gene expression.

This molecular characterization was expected to provide insight into how the enzymes evolved for nitroarene degradation. The availability of the cloned genes will also allow the construction of recombinant strains that overexpress the dioxygenase proteins for subsequent study of the oxygenase reactions. An understanding of the oxidative transformations catalyzed by the enzymes may reveal other uses for the enzymes in the biodegradation of nitroaromatic compounds and biocatalytic transformation of substituted aromatic compounds.

Methods

Bacterial strains and growth conditions. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth or LB agar unless otherwise indicated. *E. coli* DH5 was used for cloning and plasmid propagation. *Comamonas* sp. strain JS765 and *Acidovorax* sp. strain JS42 were grown in minimal salts medium (MSB; (32)) containing succinate (10 mM) and Balch's vitamin solution (7) without thiamine. For plasmid selection and maintenance, antibiotics were

added to the growth medium at the following concentrations unless otherwise noted: for *E. coli*, ampicillin 100 μg/ml, kanamycin 100 μg/ml, chloramphenicol 34 μg/ml, tetracycline 15 μg/ml, gentamicin 15 μg/ml; for JS765 and JS42: kanamycin 50 μg/ml, chloramphenicol 30 μg/ml, tetracycline 20 μg/ml, gentamicin 15 μg/ml.

DNA manipulations. Standard methods were used to manipulate plasmids and DNA fragments (26). Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs (Beverly, Mass.). Plasmids were isolated with spin miniprep kits (Qiagen, Chatsworth, Calif.), and chromosomal DNA was prepared using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.). DNA fragments were excised and purified from agarose gels using Qiaquick gel extraction kits (Qiagen). PCR was performed with an Expand Long Template PCR system (Roche, Indianapolis, Ind.). Southern hybridizations and colony blots were carried out using standard procedures (26).

Cloning of the nitrobenzene dioxygenase gene cluster. A genomic library of strain JS765 was generated by cleaving total DNA from strain JS765 then ligating those fragments with the pUC18 cloning vector (36). The library was used to transform competent *Escherichia coli* DH5α, and transformants were screened for the presence of the dioxygenase genes by colony hybridization (26). A DNA fragment complementary to the large oxygenase subunit was used as a probe to identify the recombinant strains that carried the NBDO genes. The genes for the dioxygenase were initially identified on an 8.9-kb *Eco*RI restriction fragment from strain JS765. Subsequent characterization showed that the nitrobenzene dioxygenase (NBDO) genes were contained within a 4.6-kb region of DNA. The coding region was subcloned into pUC18 and the resulting plasmid designated pDTG927. The nucleotide sequence of the region was determined and analyzed using various software packages.

Whole cell biotransformations. *E. coli* DH5α(pDTG927) was grown in Luria Bertani medium (16) and treated with 100 μM IPTG to induce synthesis of NBDO. Four hours after induction with IPTG, the cells were harvested, washed in phosphate buffer (20 mM, pH 7.5), and resuspended in buffer containing 20 mM glucose. The cell suspension was transferred to 125 ml baffled shaker flasks (final A_{600} 2.0, final volume 20 ml) then incubated with various aromatic compounds (100 μM). Alternatively, *E. coli* strains expressing wild-type and mutant dioxygenase genes were grown aerobically at 37° C in minimal medium with glucose (20 mM) as the sole carbon source, 1 mM thiamine, and ampicillin (200 μg/ml). Exponentially growing cultures were harvested by centrifugation when they reached an optical density of 1.6 to 1.8 (A_{660}), resuspended in 40 mM phosphate buffer (pH 7.3), with 20 mM glucose to an optical density of 1.6 to 1.8 (A_{660}), and 25-ml volumes were dispensed into 125-ml Erlenmeyer flasks. Cell suspensions were incubated at 30° C with shaking (200 rpm) for 6 hours with 0.1% (wt/vol) naphthalene or 4-nitrotoluene (4NT), 0.1% (vol/vol) nitrobenzene, 2-nitrotoluene (2NT), or 3-nitrotoluene (3NT), or 16 hours with 0.05% (wt/vol) 2,4-dinitrotoluene (2,4-DNT) or 2,6-dinitrotoluene (2,6-DNT). The reaction supernatants were extracted with ethyl acetate, dried under vacuum, and the residue was dissolved in an appropriate solvent. The compounds were derivatized with butyl boronate or bis(trimethysilyl)-trifluoroacetamide prior to analysis by GC-MS. Alternatively, samples were collected at appropriate intervals for HPLC and nitrite analysis (1) to monitor the progress of reaction. Reaction products present in culture supernatants were analyzed by reverse phase HPLC on a Supelcosil LC-ABZ+Plus column (Supelco, Bellefonte,

Penn) or on a Zorbax SB-C18 column (250 mm x 4.6 mm; Agilent Technologies, Palo Alto, CA). Naphthalene *cis*-dihydrodiols were purified by preparative-layer liquid chromatography using 0.1-mm-Silica Gel $60F_{254}$ plates (EMD Chemicals, Gibbstown, NJ) as previously described (21). Enantiomeric composition of naphthalene *cis*-dihydrodiol was determined using a Beckman Coulter System Gold 125 HPLC system with model 168 diode array multiwavelength detector (Beckman Coulter, Inc., Fullerton, CA) and a Chiracel OJ column (Chiral Technologies, Inc., Exton, PA.) as previously described (25).

Measurement of nitrobenzene and 2,6-DNT oxidation rates. *E. coli* strains expressing wild-type and mutant dioxygenase genes were grown in minimal medium and harvested as described above. Cells were resuspended in 40 mM phosphate buffer (pH 7.3), with 20 mM glucose to an optical density of 1.6 to 1.8 (A_{660}), and 25-ml volumes were dispensed into 125-ml Erlenmeyer flasks. Nitrobenzene was added from 0.2 and 1 M methanol stock solutions to final concentrations ranging from 12.5 μM to 15 mM. 2,6-DNT was added from a 50 mM methanol:acetone (50:50) stock solution to final concentrations ranging from 25 to 1,000 μM. Cultures were incubated at 30° C with shaking (200 rpm), and 1-ml volumes were periodically withdrawn and immediately frozen on dry ice during the initial 0 to 3 hours. Samples were stored at -20° C until assayed. Nitrite in clarified sample supernatants was determined as described previously (1). Total protein was determined by the Bradford assay (2) after resuspending cell pellets in 100 mM NaOH and boiling for 10 minutes. Bovine serum albumin was used as the standard. Kinetic coefficients of nitrite formation resulting from nitrobenzene and 2,6-DNT oxidation were estimated by non-linear regression with the Michaelis-Menten equation using Microsoft Excel Solver (31).

Protein purification. The oxygenase, ferredoxin and reductase components of 2NTDO and the oxygenase component of NBDO were purified by standard column chromatography using an automated FPLC system (Bio-Rad Laboratories, Hercules, CA). Chromatography columns and column resins were from Amersham Biosciences, Piscataway, NJ, except for the ceramic hydroxyapatite, (Bio-Rad). *Acidovorax* sp. strain JS42 was used for the isolation of $oxygenase_{2NT}$ and $ferredoxin_{2NT}$. DH5α(pDTG800), which carries the *ntdAaAbAcAd* genes from JS42 on pUC18 (19), was also used for the purification of these proteins using the same method. *E. coli* JM109(DE3)(pDTG871) was used for the isolation of $reductase_{2NT}$. The expression clone pDTG871 was constructed by amplification of the gene encoding $reductase_{2NT}$ (*ntdAa*) from plasmid pDTG800 using PCR and the following primers: 5'-*ntdAa*, 5'-GATCCATATGGAACTGGTAGTAGAACCCCTC-3', and 3'-*ntdAa*, 5'-GATCAAGCTTCAGACGCCGCTGGGATAGAACGC-3'. The 1 kb fragment was cloned into *Xcm*I-digested pTAV1. The resulting plasmid, pDTG870, and vector pT7-7 (34) were digested with *Nde*I and *Hind*III. Insertion of *ntdAa* into pT7-7 gave plasmid pDTG871, in which *ntdAa* is under the control of the T7 promoter. *E. coli* DH5α(pDTG927), which carries the *nbzAaAbAcAd* genes from JS765 in pUC18 (see above), was used for the isolation of $oxygenase_{NBZ}$. Cell extracts were prepared by allowing frozen cell suspensions to thaw on ice. DNase I was added to a final concentration of 0.01 mg/ml. Cell suspensions were passed through a chilled French pressure cell, maintaining an internal cell pressure of approximately 20,000 psi. Cell debris and membranes were removed by centrifugation at 145,000 x g for 60 minutes at 6^oC. The resulting cell extracts were used immediately for enzyme purification. Details of the column

chromatography methods can be found in the published report (22). All purified proteins were frozen in liquid nitrogen and stored at –70˚C.

Site-directed mutagenesis. Site-directed mutagenesis of *ntdAc*, which encodes the subunit of 2NTDO, was performed with the Altered Sites II *in vitro* Mutagenesis System in accordance with the manufacturer's protocol (Promega Corp., Madison, WI). A 1.1-kb *Kpn*I-*Bam*HI fragment carrying the 3' half of *ntdAc* and the 5' half of the *ntdAd* gene from pDTG850 was cloned into *Kpn*I-*Bam*HI-digested pALTER-1 (Promega Corp.). The resulting plasmid, pDTG840, was used as the template for mutagenesis. Mutagenic oligonucleotides were designed with a silent mutation that introduced a new restriction site within the gene to facilitate screening for clones carrying the desired mutation. Oligonucleotides used for generating *ntdAc* mutations are as follows:

W314F 5'-GCTACCTTCAAGGTC**TTC**AACCCGATCGATGAA-3' (*Bbs*I);
N258V 5'- GACTACTACTCCGGT**GTC**TTCAGCGCTGATATG-3' (*Bbs*I);
I350T 5'- GCGGCTCAGCGCAGT**ACT**GGACCAGCAGGATTC-3' (*Sca*I);
I350F 5'-CGCGGCTCAGCG**A**AG**CT**TCGGACCAGCAGG-3' (*Hin*dIII).

Mutagenesis of *nbzAc*, which encodes the subunit of NBDO, was carried in a similar way. Plasmid pKSJ2, the master template for mutagenesis, was constructed by subcloning a 949-bp *Pst*I-*Kpn*I DNA fragment that encodes the C-terminal portion of the subunit of NBDO and the N-terminal part of the subunit of NBDO into *Pst*I-*Kpn*I-digested pALTER-1. Phosphorylated oligonucleotides were synthesized by MWG Biotech Inc. (Greensboro, NC). Oligonucleotides used for generating *nbzAc* mutations are as follows:

N258V 5'-CTACTACTCCGGT**GT**CTTCAGCGCTGA-3' (*Bbs*I)
F293H 5'-ACGGATTTACCG**G**AGC**CA**TCTGAACGGCAC-3' (*Nla*IV)
F293I 5'-GGATTTACCGCAG**TATA**CTGAACGGCACGA-3' (*Acc*I)
F293Q 5'-GATTTACCGCAGC**CAG**CTGAACGGCACG-3' (*Pvu*II)
I350F 5'-CGCGGTTCAGCG**A**AG**CTT**CGGACCAGCAGG-3' (*Hind*III)
I350T 5'-GTTCAGCGCAGTA**CT**GGACCAGCAGGAT-3' (*Sca*I)

E. coli ES1301 *mut*S was made competent according to the protocol provided with the Altered Sites kit (Promega Corp.). The nucleotide sequences of both strands of the entire insertions in pALTER-1 were determined for each mutant. Fluorescent automated DNA sequencing was carried out by the University of California, Davis DNA sequencing facility with an Applied Biosystems 3730 automated DNA sequencer. After verifying the mutation by restriction digestion and sequence analysis, each 0.8-kb *Kpn*I-*Mfe*I fragment carrying a specific mutation in 2NTDO was individually cloned into *Kpn*I-*Mfe*I-digested pDTG850 to regenerate the complete *ntd* operon. After restriction analysis and nucleotide sequencing of both strands of the insertion in pKSJ2 to verify the desired mutations, the 949-bp *Pst*I-*Kpn*I DNA fragments were individually cloned into pKSJ4 to regenerate the complete *nbz* operon. This subcloning step was followed by restriction digestion to verify the presence of the mutations. The resulting plasmids were introduced into DH5 for expression studies, and DH5(pUC18) was used as a vector control.

Construction of *ntdAa-lacZ* reporter fusion. A 241-bp DNA fragment encompassing the entire *nbzAa* promoter region including 135-bp upstream of the putative LysR-type binding site was generated by PCR using the primers, NBDOPRO1 (5'-GGGGTACCCCTTTAAGTGAATTGCTGACGGCAGG-3') and NBDOREV (5'-GCTCTAGAGCGCAAGCTCTTTTTTCAGTTGTCTC-3'). This 241-bp fragment was ligated to *Kpn*I-*Xba*I-digested pMW24-RPOS to generate pDTG928. *Not*I-digested pDTG928 was ligated to *Not*I-digested pUTminiTn*5*-Gm (3) to yield pDTG931. This plasmid is mobilizable and contains the *nbzAa-lacZ* transcriptional fusion and a gentamicin resistance gene within the mini-Tn*5*. Since the sequence of *nbzR-nbzAa* region is identical to the *ntdR-ntdAa* region, a single fusion could be constructed for the study of both the *nbz* and *ntd* gene clusters. Introduction of the *ntdAa-lacZ* (as it will be referred to from now on) fusion into JS765 and JS42 was done by mating with *E. coli* S17-1(pDTG931). Gentamicin-resistant colonies were selected and screened for the ability to form blue colonies on agar plates containing X-gal. Random single Tn*5* insertions in the resulting transconjugants were confirmed by PCR and Southern blot analyses. In this way, expression of the same *ntdAa-lacZ* fusion could be analyzed in a number of different strains under a variety of inducing conditions. Strains carrying the fusion were still capable of growth with nitrobenzene and 2NT, respectively, similar to their wild-type counterparts. Strains JS765 and JS42 carrying the fusion (and also JS42-R; see below) were used for analysis of *lacZ* expression. Derivatives of the naphthalene degrading strain *P. putida* G7 (4), the 2,4-DNT degrading strain *Burkholderia cepacia* JS872 (17), and *E. coli* carrying single copies of the *ntdAa-lacZ* fusion were generated in the same way and were used to monitor gene expression in these alternative strain backgrounds.

Construction and complementation of a JS42 *ntdR* mutant strain. *dntR* is the *lysR*-type regulatory gene from *Burkholderia* sp. strain DNT (33) that is located upstream of the 2,4-DNT dioxygenase genes. At the amino acid sequence level, *dntR* is 97% identical to *ntdR* and *nbzR*. A 7.0-kb pJS31 *Bgl*II fragment containing *dntR* was cloned into *Bam*HI-digested pRK415 to form pRK415-DntR. This plasmid was digested with *Bam*HI (unique site in *dntR*) and ligated with a 2.4-kb *Bam*HI fragment containing the kanamycin resistance cassette from pHP45Ω-Km (5). The resulting plasmid containing *dntR*::Km^R was designated pDTG957. pDTG957 was mobilized into JS42 carrying the *ntd-lacZ* fusion as described above. Kanamycin-resistant colonies were selected and a tetracycline-sensitive mutant, JS42-R, was identified. This mutant contained *ntdR*::KmR in place of *ntdR*, as shown by PCR and Southern analyses with a *ntdR* probe, and it was unable to grow on 2NT. A 0.9-kb DNA fragment containing *ntdR* was generated by PCR amplification with pDTG925 as template. This fragment was ligated to *Kpn*I-digested pBBR1-MCS (12) to form pNtd1. Introduction of pNtd1 into JS42-R was done by mating as described above. Chloramphenicol-resistant colonies were selected. The presence of pNtd1 in JS42-R was confirmed by plasmid purification and transformation of *E. coli* DH5α. The complemented strain JS42-R(pNtd1) grew on 2NT as sole carbon and energy source. Analogous clones carrying the LysR-type regulators NahR and NagR from the naphthalene degrading strains *Pseudomonas putida* G7 and *Ralstonia* sp. strain U2, respectively, were generated by PCR amplification of the *nagR* and *nahR* genes, which were then cloned into pBBR1-MCS, and mated into JS42-R to test for complementation by these related regulatory proteins.

Construction of a JS42 *ntdAc* mutant strain. pDTG850 (pUC18 carrying *ntdAaAbAcAd*; (21)) was digested with *Kpn*I, which cuts once in the *ntdAc* gene, and blunt ends were generated with T4 polymerase. A 1.3 kb DNA fragment carrying the kanamycin Genblock cassette (Pharmacia) was gel purified using the Qiagen kit, and ligated into linearized pDTG850. A Ap^R, Km^R, Tc^S clone with the appropriate insert was verified by digestion with *Pst*I and *Bam*HI. The *Sac*I-*Eco*RI fragment from pDTG850-Km (carrying *ntdAaAbAc::*Km*Ad*) was moved into *Sac*I-*Eco*RI-digested pRK415 (11). Tc^R, Km^R, Ap^S strains were screened for the correct *Sac*I-*Eco*RI insert. The plasmid, pRK415-850-Km was used to transform *E. coli* S17-1 (29). *E. coli* S17-1(pRK415-850-Km) was mated with JS42 on LB plates overnight at 30°C. Mating mixes were resuspended in MSB medium, washed once, and plated onto selective medium: MSB containing 10 mM succinate, 50 μg/ml kanamycin and vitamins. Kanamycin resistant strains were grown in liquid medium of the same composition through several transfers, and plated on plates of the same type. Km^R, Tc^S colonies were identified and tested for the ability to grow with 2NT. JS42-*ntdAc*::Km was unable to grow on 2NT, but retained catechol dioxygenase activity. PCR was used to confirm the insertion of the kanamycin resistance gene. We cloned the *ntdAcAd* genes into pBBR1-MCS, introduced the clone into JS42-*ntdAc*::Km by mating from *E. coli*, and demonstrated that the mutation can be complemented.

Primer extension analysis. Primer extension analysis of the *nbz* and *ntd* operons was carried out as described previously (35). RNA was prepared from JS765 and JS42 using the Trizol reagent (Life Technologies, Grand Island, N.Y.). RNA was extracted from cultures grown to an optical density at 660 nm (OD_{660}) of 0.25. The initial OD_{660} was 0.05. The primer used for extension was 5'-GCCGCAGCGGCCCGACATGCA-3'. The primer was 5' end-labeled using [γ^{32}-P]dATP and a KinaseMax kit (Ambion, Austin, Tex.). The ^{32}P-labeled primer was annealed to 10 to 20 μg of JS765 or JS42 RNA and extended using a First-Strand cDNA synthesis kit (Amersham, Piscataway, N.J.). DNA sequences were obtained using pDTG925 as a template and the same primer used for primer extension. Sequencing was with α-^{35}S-dATP and a Sequenase, version 2.0, DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio). DNA fragments were resolved on 8 M urea-8% polyacrylamide gels.

RT-PCR. Total RNA was isolated from JS765 and JS42 grown in MSB with succinate or succinate plus salicylate, NB (JS765) or 2NT (JS42) by using a Rneasy total RNA kit (Qiagen). Purified RNA was treated with Rnase-free Dnase (Qiagen) to remove DNA contaminants. RT-PCR was carried out with an Access RT-PCR kit (Promega). The following primer pairs were used: P760F (5'-TGCCTAGCGATGCGGAAATG-3') and P420R (5'-TCTCGGACATGTTCTGCAAC-3') for the *nbzAaAb* region and P760F and PprobeR (5'-ACGTGGTAGCCGTCACCTAC-3') for the *nbzAaAbAc* region.

Analysis of *lacZ* expression. Wild-type and mutant strains of JS765 and JS42 containing *ntdAa-lacZ* were grown in MSB supplemented with 10 mM succinate, appropriate antibiotic(s), vitamins, and potential inducing compounds: nitrobenzene, mononitrotoluenes, DNTs, ADNTs, TNT, catechol and 3-methyl catechol, salicylate, anthranilate (final concentration, 100 or 500 μM). Cultures were inoculated to an initial OD_{660} of 0.03 to 0.06 and β-galactosidase activity was measured by the method of Miller (16) when the OD_{660} reached between 0.2 and 0.3.

Results and Discussion

Cloning and sequence analysis of nitroarene dioxygenase gene clusters. The genes encoding nitrobenzene dioxygenase were cloned from strain JS765 and the nucleotide sequence determined. A clone (pDTG925) carrying a 8.9-kb *Eco*RI fragment was identified from a JS765 clone bank by colony hybridization. The hybridization probe was a PCR product obtained by amplification of JS765 genomic DNA using primers generated based on regions of conserved dioxygenase large subunit genes. Five complete and one partial open reading frame were found in the 4.6-kb DNA fragment subcloned in pDTG927 (Figure IV-1).

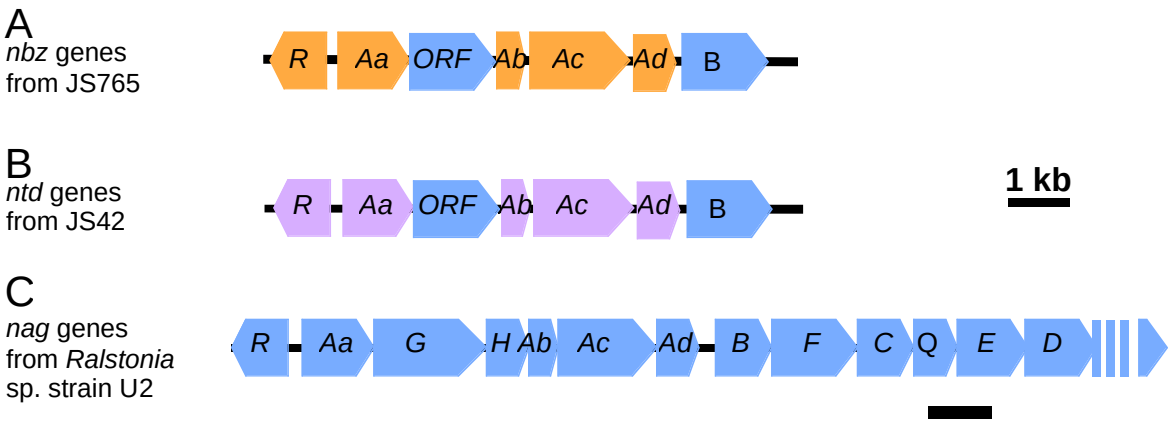


Figure IV-1. Organization of the genes encoding NBDO from JS765 compared to those encoding 2NTDO and NDO. A) Sequenced fragment of genomic DNA from *Comamonas* sp. JS765 containing the *nbz* genes. The *nbz* genes from JS765 are orange. B) The *ntd* genes encoding 2NTDO from *Acidovorax* sp. JS42 are lavender. Genes that appear to be nonfunctional remnants of the *nag* gene cluster are shown in blue. C) Genetic organization of the naphthalene catabolic genes (*nag*) from *Ralstonia* sp. strain U2.

The predicted amino acid sequences share significant identity with polypeptides from three component dioxygenase systems. The strongest identity was found with the protein components of nitroarene dioxygenases (2NTDO and DNTDO) and the naphthalene dioxygenase from strain U2 (19, 33, 38). The function of four gene products was predicted from their similarity to previously described proteins: *nbzAa* (reductase), *nbzAb* (ferredoxin), *nbzAc* and *nbzAd* (oxygenase □- and □-subunits, respectively). The partial *nbzB* gene appears to encode a *cis*-dihydrodiol dehydrogenase based on sequence comparisons, but such an enzyme is unnecessary for nitrobenzene degradation, and appears to have been acquired from a *nag*-like pathway cluster. The remaining open reading frame (ORF) has high sequence homology with the 5'-end of the *nagG* gene from *Ralstonia* sp. strain U2. *nagG* encodes the □-subunit of the multicomponent salicylate 5-hydroxylase, which catalyzes the conversion of salicylate to gentisate in the naphthalene degradation pathway of that strain (37). Since neither JS42 nor JS765 is capable of growth on salicylate, it appears that the truncated *nagG* homologue was carried along when these strains obtained ancestral naphthalene degradation gene cluster by horizontal transfer. The gene order in the *nbz* cluster is identical to that of the *ntd* genes, which encode 2NTDO from *Acidovorax* sp. JS42 (Figure IV-1). Although the two sets of genes had

very high sequence identities (>95% identity), the encoded dioxygenases have different substrate specificities. A comparison of the amino acid identities of the catalytic subunits of related dioxygenases is shown in Table IV-1. Details of this study have been published (14).

Table IV-1. Amino acid sequence identity matrix comparing the catalytic (□) subunits of NBDO and related dioxygenases

Strain/Protein	**JS765/NbzAc**	**JS42/NtdAc**	**DNT/DntAc**	**U2/NagAc**
JS765/NbzAc	100	95	87	88
JS42/NtdAc		100	88	90
DNT/DntAc			100	88
U2/NagAc				100

Percent identities of pair-wise alignments are shown for protein sequences from NBDO, 2NTDO, 2,4-DNTDO, and NDO from *Comamonas* sp. JS765, *Acidovorax* sp. JS42, *Burkholderia* sp. DNT, and *Ralstonia* sp. U2, respectively.

Regulation of nitroarene dioxygenase gene clusters. Transcribed divergently from each dioxygenase gene cluster is a LysR-type transcriptional regulatory gene (*nbzR* and *ntdR*; Figure IV-1). The complete *ntdR* gene was PCR amplified from JS42, cloned and sequenced. NbzR and NtdR are identical in sequence and are homologous to the well-studied NahR (Table IV-2), which is a transcriptional activator of the naphthalene degradation genes in *Pseudomonas putida* G7 (27, 28). They also share 98% amino acid sequence identity with NagR, the transcriptional regulator of the naphthalene degradation gene cluster from *Ralstonia* sp. U2 (9).

Table IV-2. Amino acid sequence identity matrix comparing the LysR-type regulators from nitroarene and naphthalene degradation pathways

Strain/Protein	**JS765/NbzR**	**JS42/NtdR**	**DNT/DntR**	**U2/NagR**	**G7/NahR**
JS765/NbzR	100	100	97	98	61
JS42/NtdR		100	97	98	61
DNT/DntR			100	99	61
U2/NagR				100	61
G7/NahR					100

Percent identities of pair-wise alignments are shown for LysR-type proteins from *Comamonas* sp. JS765, *Acidovorax* sp. JS42, *Burkholderia* sp. DNT, *Ralstonia* sp. U2, and *P. putida* G7.

Previous studies suggested that the NBDO genes were inducible and the 2NTDO genes were constitutive (8, 18). RT-PCR was used to demonstrate that the *nbz* and *ntd* genes are each co-transcribed in a single operon. Each transcript consisted of five genes transcribed in one

direction, with the regulatory gene transcribed in the opposite direction. For each gene cluster, two sets of primers were used to demonstrate that genes *Aa*, Orf2, and *Ab* are cotranscribed, and that these three genes are cotranscribed with the *Ac* gene (Figure IV-2). The results demonstrate that a single promoter upstream of the *Aa* gene controls expression of the operon.

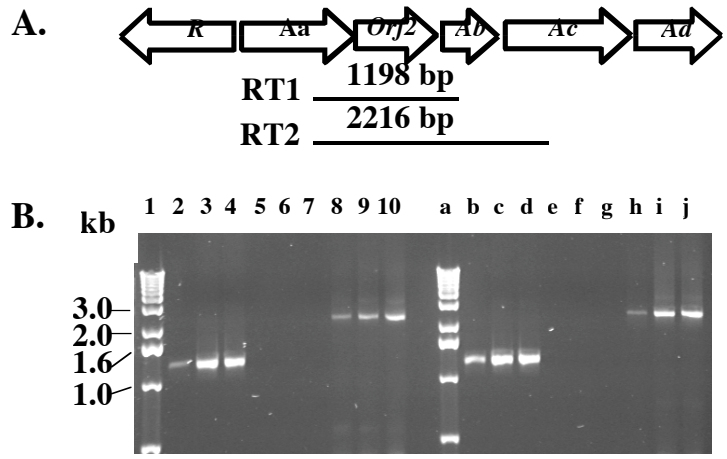


Figure IV-2. RT-PCR analysis of the operon structure of the *nbz* and *ntd* genes. A) The genetic organization of the *nbz* genes from strain JS765 and *ntd* genes from strain JS42. Arrows indicate the direction of transcription. The locations of primer sets and the amplified DNA fragments for RT-PCR are designated RT1 and RT2. B) RT-PCR amplification of the *nbz* (lanes 2-10) and *ntd* (lanes b-j) gene clusters. Lanes 2, 5, 8, RT-PCR products from total RNA from succinate-grown JS765; lanes 3, 6, 9, RT-PCR products from total RNA from succinate plus NB-grown JS765; lanes 4, 7, 10, RT-PCR products from total RNA from succinate plus salicylate grown JS765; lanes b, e, h, RT-PCR products from total RNA from succinate grown JS42; lanes c, f, i, RT-PCR products from total RNA from succinate plus 2NT-grown JS42; lanes d, g, j, RT-PCR products from total RNA from succinate plus salicylate-grown JS42. Samples in lanes 5-7 and e-g were without RT. Lanes 1-7 and b-h show amplifications performed with the RT1 set and lanes 8-10 an d 18-20 with the RT2 set. Lanes 1 and a contain 1 kb ladder.

The nucleotide sequences of the intergenic region, including a putative NahR binding site, in the *nbz*, *ntd* and *nag* dioxygenase gene clusters are identical (Figure IV-3A). Primer extension was used to identify the transcriptional start sites, which were found to correspond to those of the *nah* and *sal* promoters in *P. putida* G7 (Figure IV-3B). The consensus NahR binding site was found in the *ntd* and *nbz* intergenic regions (Figure IV-3A), suggesting that NtdR and NbzR, which are homologues of NahR, may bind in a similar location at their respective operators. Results of both RT-PCR and primer extension experiments suggested that the genes were induced in the presence of nitroarene compounds.

A

```
U2      ATGCCTCACCATTATTCATGCTGGTGATTTTAACTATCAGACTTGATCTATAGCGCTATACCGATCGACGCGCCAGAATCGCAGCCATTC
JS765   ATGCCTCACCATTATTCATGCTGGTGATTTTAACTATCAGACTTGATCTATAGCGCTATACCGATCGACGCGCCAGTATCGCAGCCATTC
JS42    ATGCCTCACCATTATTCATGCTGGTGATTTTAACTATCAGACTTGATCTATAGCGCTATACCGATCGACGCGCCAGTATCGCAGCCATTC
R34     CACCCTCACCATTATTCATGCTGGTGATTTTAACTATCAGACTTGATCTATAGCGCTATACCGATCGACGCGCTAGAATCGCAGCCATTC
DNT     CACCCTCACCATTATTCATGCTGGTGATTTTAACTATCAGACTTGATCTATAGCGCTATACCGATCGACGCGCTAGAATCGCAGCCATTC
G7sal   GGCCGGGCGCAATATTCATGTTGATGATTTATTATATATCGAGTGGTGTATTTATCAATATTGTTTGCTCCGTTATCGTTATTAACAAGT
G7nah   GACGATTCGCAGTATTCACGCTGGTGATAAACAAATTCAACTATGCTTTATTGACAAATAAAAGCACGCTCACCATCATCGCGAATACAA
                                                          -35                  -10
Consensus     CGCA-TATTCA-G-TG-TGAT--A--A--T
NahR binding
```

B C T A G 1 2

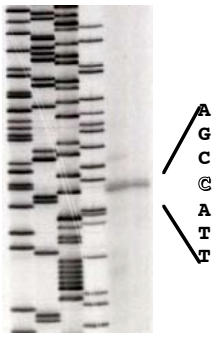


Figure IV-3. Promoter analysis of the *nbz* and *ntd* operons. A). Alignment of the nitroarene and naphthalene dioxygenase gene promoters from *Ralstonia* sp. U2, *Comamonas* sp. JS765, *Acidovorax* sp. JS42, *Burkholderia cepacia*, R34, *Burkholderia* sp. DNT, and *P. putida* G7. The consensus NahR binding sequence is shown below the alignment. Conserved nucleotides are shaded. The –35 and –10 sites are underlined. Identified transcription start sites are highlighted. B) Primer extension analysis of the *nbz* and *ntd* transcripts from JS765 and JS42, respectively. The sequencing ladder is shown at the left with primer extension products from strain JS765 (lane 1) and from strain JS42 (lane 2). The transcription start site is shown in outlined font.

In order to further study the transcriptional regulation of these two gene clusters, merodiploid strains of JS765 and JS42 containing the wild-type dioxygenase gene cluster and a chromosomal *ntdAa-lacZ* fusion were constructed. Analysis of β-galactosidase activity in these strains indicated that dioxygenase gene expression is induced in the presence of the nitroaromatic compounds nitrobenzene (NB), 2NT, 3NT, and 4NT, 2,4-DNT and 2,6-DNT, 2-amino-4,6-DNT, and 4-amino-2,6-DNT (ADNTs) (Figure IV-4). We have also demonstrated induction by 2,4,6-TNT in JS42 (Figure IV-5). The finding that ADNTs and TNT induce the dioxygenase genes is significant in light of our current strategy to degrade TNT via initial ring reduction to ADNT isomers, followed by ring hydroxylation of the ADNTs by nitroarene dioxygenases.

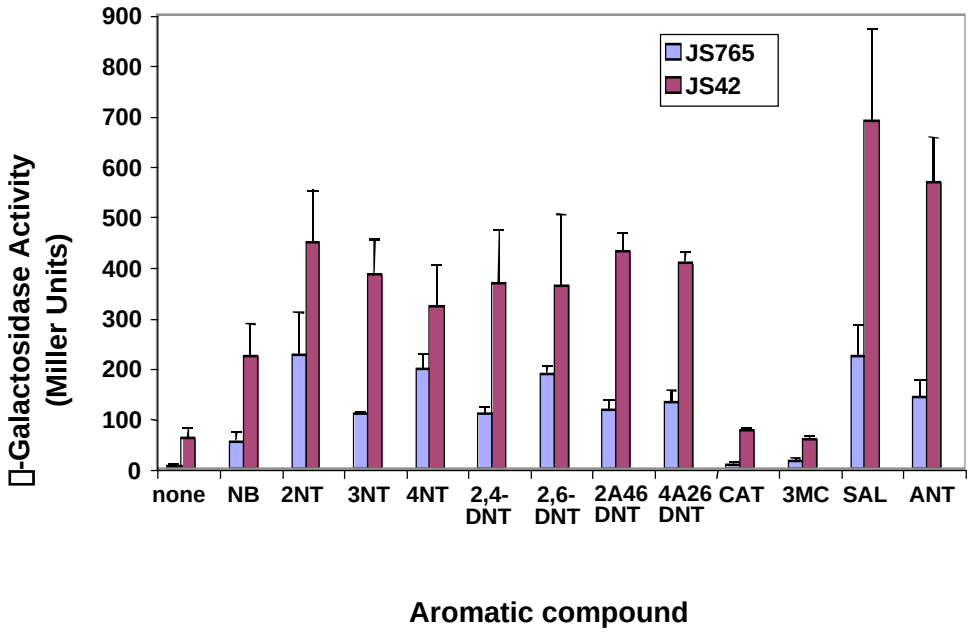


Figure IV-4. -Galactosidase activity expressed from the *ntdAa-lacZ* fusion in strains JS42 and JS765 after growth in the presence of various potential inducing compounds. NB, nitrobenzene; NT, nitrotoluene; DNT, dinitrotoluene; cat, catechol; sal, salicylate; ant, anthranilate (500 M each); ADNT, aminodinitrotoluene (100 M).

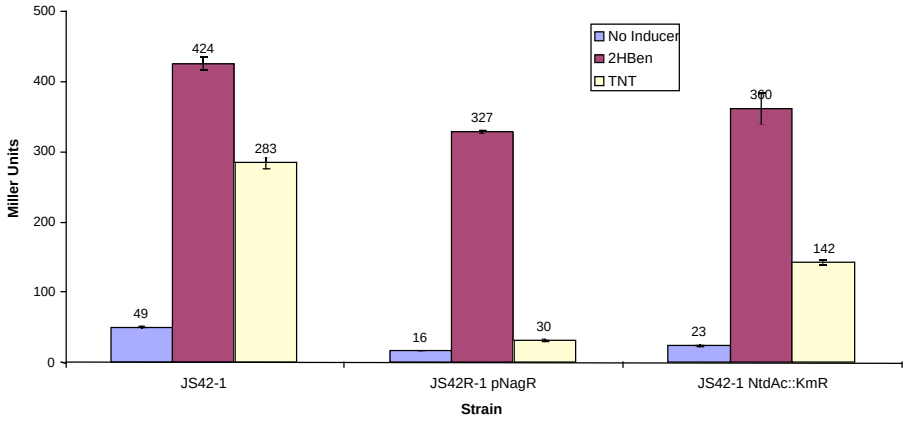


Figure IV-5. -Galactosidase activity expressed from the *ntdAa-lacZ* fusion in strains JS42, JS42-R (*ntdR* regulatory mutant) complemented with *nagR in trans*, and JS42 with a *ntdAc* mutation after growth in the presence of no inducer, 500 M salicylate (2-hydroxybenzoate; 2Hben), or 500 M 2,4,6-TNT.

Salicylate (SAL) and anthranilate (ANT), were also good inducers of the *nbz* and *ntd* genes (Figure IV-4). This finding provides further evidence that nitroarene dioxygenase gene clusters evolved from an ancestral naphthalene dioxygenase gene cluster because salicylate is the natural inducer of the naphthalene dioxygenase genes in *P. putida* G7 and *Ralstonia* sp. U2 (9,

28). The best inducers for the *nbz* genes in JS765 were 2NT, salicylate, 4NT, and 2,6-DNT. The best inducers of the *ntd* genes in JS42 were salicylate, anthranilate, and 2NT. Although both sets of genes were inducible, it is clear from the data that the basal level of expression in the absence of inducer was higher in JS42 than in JS765. This result may explain the previous results that suggested constitutive expression of 2NTDO in JS42 (8). The products of nitrobenzene and 2NT oxidation, catechol (CAT) and 3-methylcatechol (3MC), did not induce *lacZ* transcription in the wild-type background (Figure IV-4), indicating that the nitroarene compounds were directly responsible for induction and metabolism was not required. This result was verified by constructing a *ntdAc* mutant of JS42 and introducing the *ntdAa-lacZ* fusion into that strain (JS42-*ntdAc*::Km). JS42-*ntdAc*::Km is unable to grow on 2NT and completely lacks 2NTDO activity. However, the *ntdAa-lacZ* fusion was regulated the same in JS42 and JS42-*ntdAc*::Km, indicating that metabolism of 2NT is not required for induction of the *ntd* promoter (Figures IV-5 and IV-6). These results indicate that JS42 has evolved the ability to grow on and degrade the man-made nitroarene compound 2NT, and has also evolved the ability to regulate the degradation genes appropriately: NtdR has the ability to detect and respond to nitroarene compounds to turn on 2NTDO gene expression.

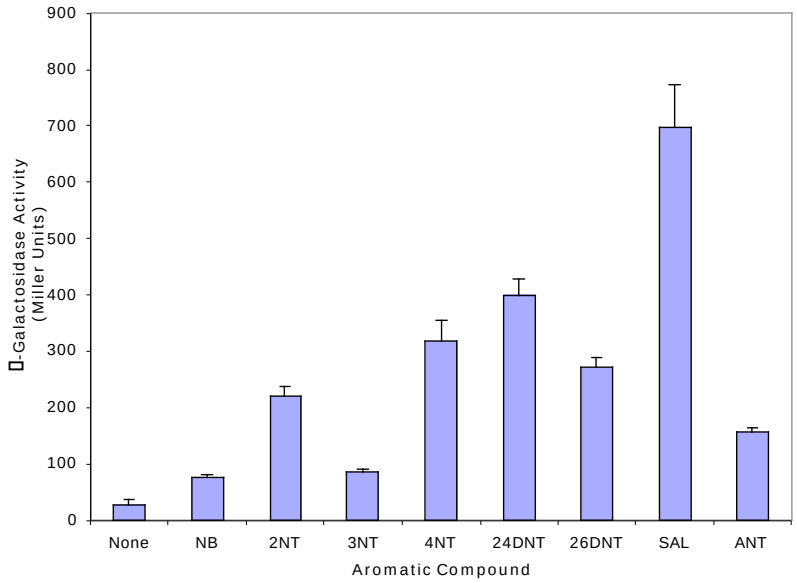


Figure IV-6. -Galactosidase activity expressed from the *ntdAa-lacZ* fusion in strain JS42*ntdAc*::Km (a strain with a *ntdAc* mutation that prevents growth on or metabolism of 2NT) after growth in the presence of no inducer or 500 M of various aromatic inducers. Abbreviations are as in Figure IV-4.

As mentioned above, a *lysR*-type regulatory gene is located upstream of each dioxygenase gene cluster. We were interested in determining whether these genes were involved in the regulation of the nitroarene genes. A mutant strain of JS42 in which the *ntdR* regulatory gene was disrupted by introduction of a kanamycin resistance gene cassette was constructed. Unlike the wild type, JS42-R was unable to grow with 2NT as sole carbon source, indicating that *ntdR* is essential for growth on 2NT. A complemented strain was generated by introducing the cloned *ntdR* gene on a broad-host-range plasmid. This strain, JS42-R(pNtd1), was able to grow

with 2NT. This result demonstrated that the *ntdR* gene was expressed *in trans* and was able to complement the *ntdR* mutation. JS42-R carries the *ntdAa-lacZ* fusion, and β-galactosidase assays indicated that the *ntd* genes in JS42-R are not induced under any conditions, demonstrating that NtdR is absolutely required for background expression as well as induction of the *ntd* genes by nitroarene compounds, salicylate, and anthranilate (Figure IV-7). Background and induced β-galactosidase levels were slightly higher in the complemented strain than in the wild type, probably due to the increased copy number of the plasmid-encoded regulatory gene.

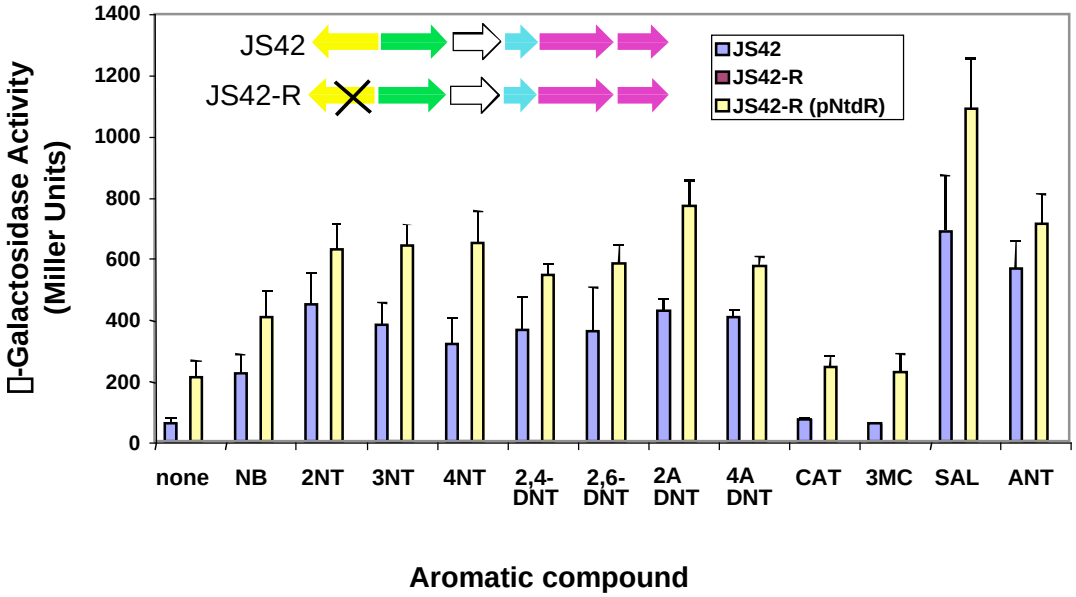


Figure IV-7. β-Galactosidase activity expressed from the *ntdAa-lacZ* fusion in strains JS42 JS42-R (*ntdR* regulatory mutant), and JS42-R complemented with *ntdR in trans* after growth in the presence of various potential inducing compounds. Abbreviations are as in Figure IV-4.

In contrast, introduction of *nagR* from *Ralstonia* sp. U2 or *nahR* from *P. putida* G7 into the JS42-R regulatory mutant was unable to complement the *ntdR* mutant for induction with nitroarene compounds. NagR mediated induction by salicylate, and NahR mediated induction by salicylate and anthraniliate (Figures IV-5 and IV-8). These results suggest that NtdR is unique in its ability to detect and respond to nitroarene inducers and that one or more of the five amino acid differences between NtdR and NagR controls nitroarene recognition.

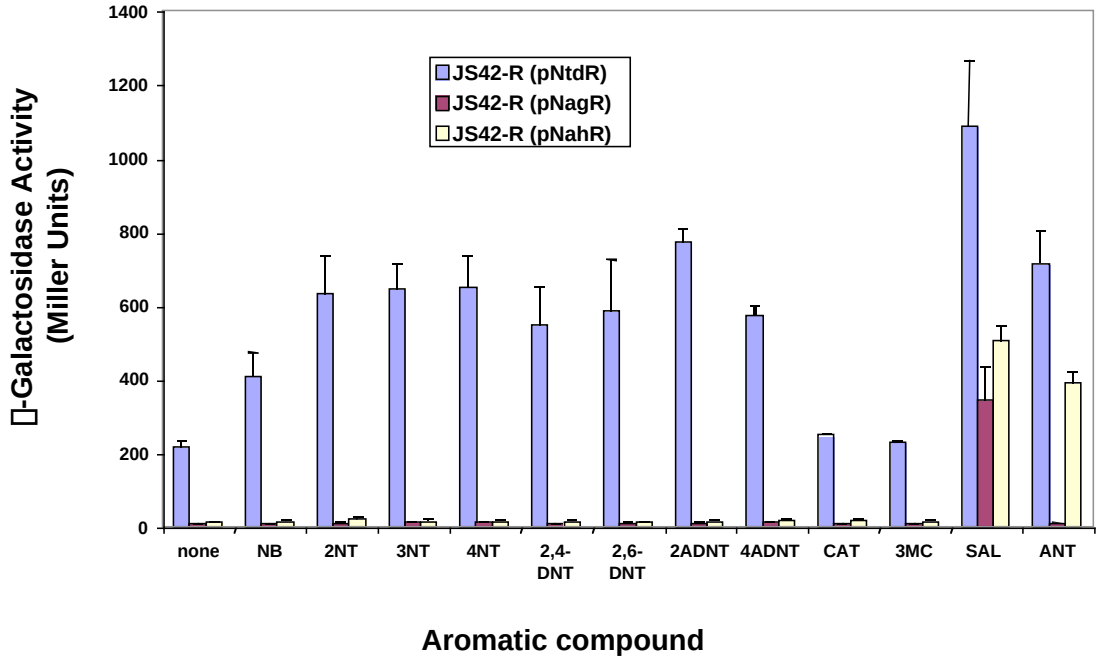


Figure IV-8. -Galactosidase activity expressed from the *ntdAa-lacZ* fusion in the JS42 regulatory mutant JS42-R complemented with NtdR, NagR (from *Ralstonia* sp. U2) or NahR (from *P. putida* G7) after growth in the presence of various potential inducing compounds. Abbreviations are as in Figure IV-4.

Results from assays with *E. coli* harboring the *ntdAa-lacZ* chromosomal fusion and a plasmid expressing *ntdR* demonstrated increased -galactosidase activity when cells were grown in the presence of salicylate and anthranilate, but not with nitroaromatic compounds or catechols. No -galactosidase was seen under any conditions when NtdR was not provided (Figure IV-9). These results indicate that NtdR was functional in *E. coli* as it mediated induction by salicylate and anthranilate but that some element of the regulation system was not functioning to mediate induction by nitroarenes.

Assays with *P. putida* G7 harboring *ntdAa-lacZ* demonstrated increased -galactosidase activity when cells were grown in the presence of salicylate, but not with nitroaromatic compounds (Figure IV-10). These results suggest that NahR in G7 can recognize the putative binding site in the *nbz*/*ntd* promoter regions. Activity was not increased in the presence of nitroaromatic compounds, suggesting that G7 lacks specific regulatory elements present in JS765 and JS42. Similar experiments were carried out in other backgrounds, including *Pseudomonas putida* PRS2000 and *Ralstonia eutropha* JMP289. When these strains carried a single copy of the *ntdAa-lacZ* fusion and *ntdR* was provided *in trans*, only salicylate and anthranilate functioned as inducers (data not shown). All of these data suggest that there is something unique about JS42 and JS765 that allows induction by nitroarene inducers, which is lacking in other bacteria.

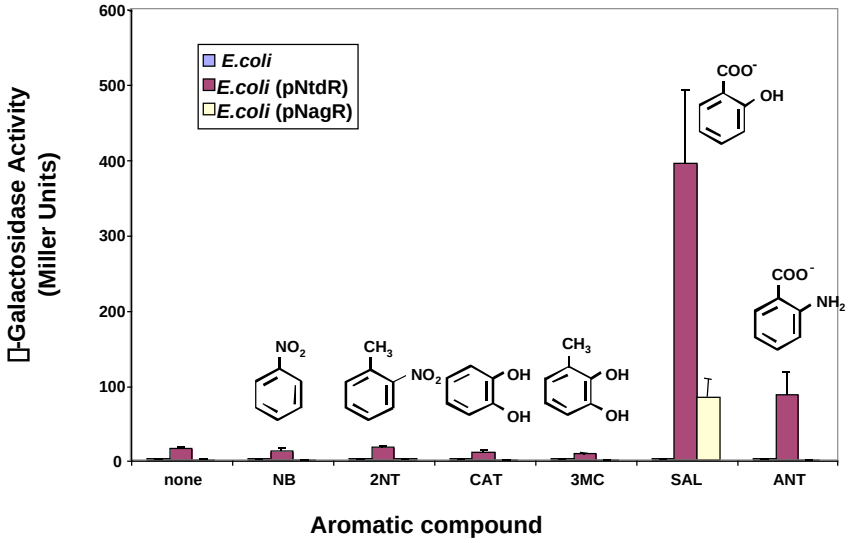


Figure IV-9. -Galactosidase activity expressed from the *ntdAa-lacZ* fusion in *E. coli* in the presence and absence of NtdR or NagR after growth in the presence of various potential inducing compounds. Abbreviations are as in Figure IV-4.

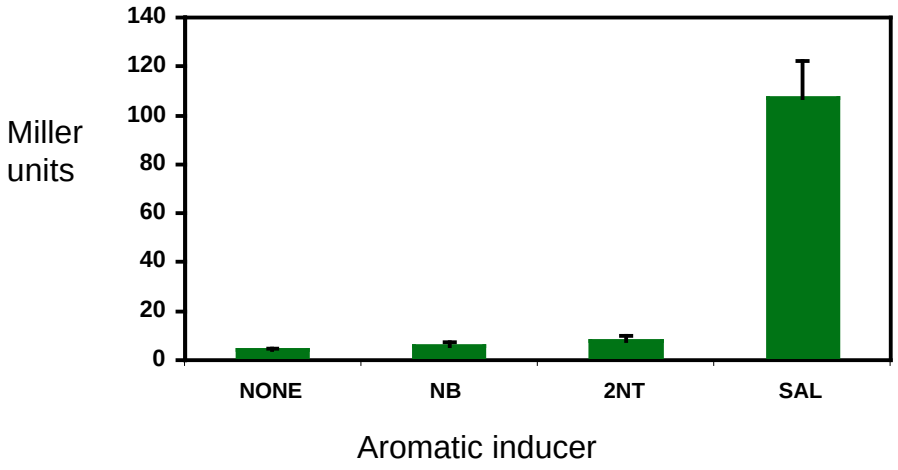


Figure IV-10. -Galactosidase activity expressed from the *ntdAa-lacZ* fusion in *P. putida* G7 after growth in the presence of various potential inducing compounds. Abbreviations are as in Figure IV-4.

Possible explanations for the inability of other bacteria to recognize nitroarene inducers include: 1) nitroarene compounds were not taken up by *E. coli*; 2) a metabolite of the nitroarene compounds was the actual inducer; 3) reduced (amino) forms of the nitroarene compounds were the actual inducers; 4) an additional regulatory protein was present in JS42 and JS765; 5) some component of the transcription system (e.g. polymerase; sigma factor) in JS42 and JS765 was different; 6) that the promoter was modified (e.g. methylated) in JS42 and JS765. To date, we

have ruled out the first three possibilities. The problem does not appear to be insufficient transport of the nitroarene substrates, because whole cells of *E. coli* carrying the dioxygenase genes on a plasmid are able to transform nitroarene substrates into catechols and nitrobenzyl alcohols. Based on the inability of catechol and 3-methylcatechol to induce, we expected that the nitroarene compounds were the actual inducers and they did not need to be converted to pathway intermediates to allow induction. We confirmed this by carrying out two additional experiments. First, we introduced the NBDO genes on pDTG927 into *E. coli* GS162R1, and still did not detect induction by 2NT (Table IV-3). And second, we generated an *ntdAc* mutant of JS42 (JS42*ntdAc*::Km) that is unable to metabolize or grow on 2NT, and introduced the *ntdAa-lacZ* fusion into it. Induction by nitroarene compounds was just as in JS42 as shown above (Figure IV-6). We also showed that the reduced forms of nitrobenzene and 2NT (aniline and *o*-toluidine) were not inducers (Table IV-3).

Table IV-3. Activity of the *ntdAa-lacZ* fusion with aminoaromatic compounds

	-galactosidase activity[a] in strain:	
Chemical added	JS42	GS162R-1 (pNtd1) (pDTG927)
None	119 ± 13	3.5 ± 0.3
Salicylate	ND [b]	47 ± 12 (13)
2NT	347 ± 46 (3)	3.2 ± 0.3 (1)
Aniline	126 ± 9 (1)	4.2 ± 0.7 (1)
o-Toluidine	155 ± 10 (1)	3.9 ± 0.5 (1)

[a] Expressed as Miller units ± standard deviation. Fold inductions (activity of cells grown with succinate + chemical inducer/activity of cells grown with succinate alone) are shown in parentheses.
[b] ND, not determined.

In an earlier study, we identified a second *lysR*-type regulatory gene in JS765 (23), and we have since verified its presence in JS42. In each strain, this gene (*cdoR*) is located in a gene cluster for catechol/3-methylcatechol degradation. Introduction of *cdoR* and *ntdR* together in *E. coli* did not allow induction of the *ntdAa-lacZ* fusion in *E. coli*, so this protein does not appear to be the missing element (Figure IV-11). We carried out a search for a missing regulatory element by introducing JS42 and JS765 clone banks into a strain of *E. coli* carrying *ntdR* and the *ntdAa* promoter fused to a promoterless kanamycin resistance gene, and selecting for kanamycin resistance in the presence of 2NT, but not in its absence. The reporter itself appeared to function properly; we were able to demonstrate salicylate-indcucible kanamycin resistance. However, we did not obtain any 2NT-inducible kanamycin resistant clones. At this time we are setting up to

carry out *in vitro* analysis of the *ntd* promoter in order to address the possibility that the difference is in the JS42/JS765 transcription machinery or in methylation patterns.

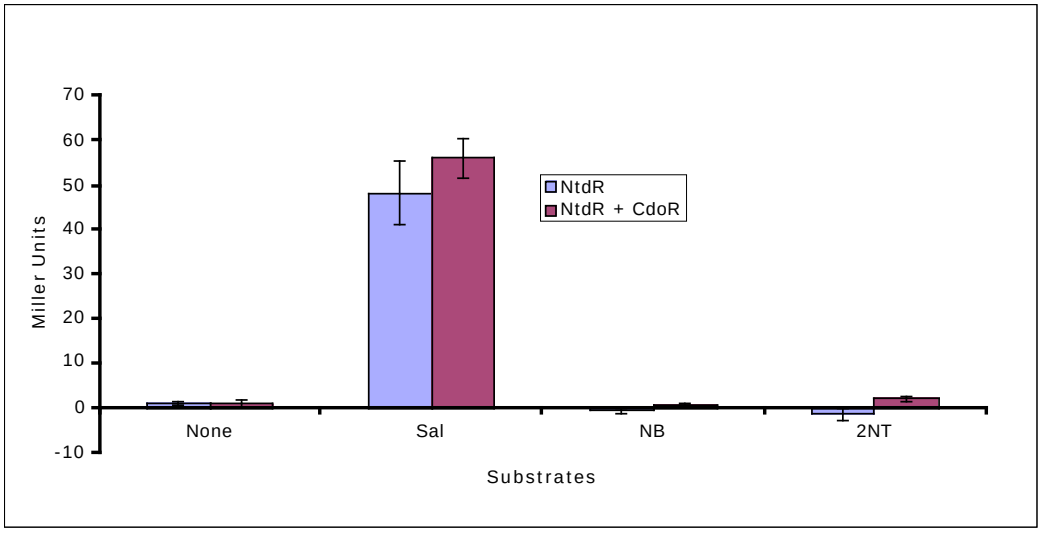


Figure IV-11. β-Galactosidase activity expressed from the *ntdAa-lacZ* fusion in *E. coli* carrying NtdR in the presence and absence of CdoR after growth in the presence of various potential inducing compounds. Abbreviations are as in Figure IV-4.

In order to probe the regulation of the DNT degradation genes, the *ntdAa-lacZ* fusion was introduced into the 2,4-DNT degrading strain *Burkholderia cepacia* JS872. As in JS42 and JS765, salicylate proved to be an inducer, indicating that the native DntR regulatory protein in JS872 recognized the *ntd* promoter (the *dntAa* and *ntdAa* promoters only differ by three base pairs). When cells were induced with 2,4-DNT, however, this substrate was only weakly inducing after several days' exposure, suggesting that DntR is not as responsive to nitroarene inducers as NtdR (Figure IV-12) These results are consistent with results of nitrite assays discussed in the first section of this report. DntR is more similar in amino acid sequence to NagR than NtdR, so this result is not unexpected. The results thus far are also consistent with modeling of the inducers salicylate and 2,4-DNT in the crystal structure of DntR from the 2,4-DNT degrading strain *Burkholderia* sp. DNT (30). This protein appears to easily accommodate salicylate but not 2,4-DNT in the inducer binding pocket. Current experiments are under way to determine the full range of inducers recognized by DntR. An additional set of studies in JS872 indicated that expression of the nitroarene degradation genes in this strain is under the control of catabolite repression by sugar and carboxylic acid substrates (Figure IV-13).

Finally, we have recently identified a mutant form of NtdR that is inducer-independent and causes constitutive expression of the dioxygenase genes (1900 Miller units in JS42 in the absence of added inducer). This protein may provide an alternative mechamism to develop a constitutively expressed engineered pathway.

Many of the details of this study have been published (15), and an additional manuscript is in preparation.

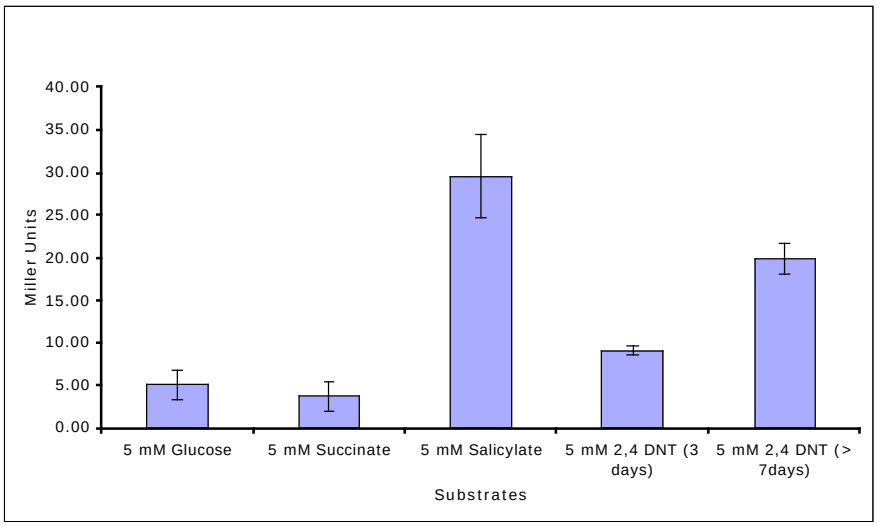


Figure IV-12. β-Galactosidase activity expressed from the *ntdAa-lacZ* fusion in *Burkholderia cepacia* JS872 after growth in the presence of various potential inducing compounds.

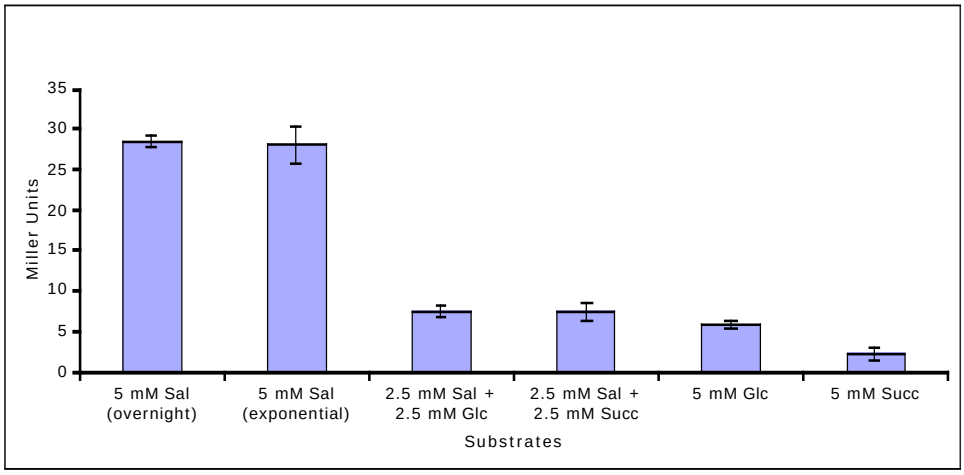


Figure IV-13. β-Galactosidase activity expressed from the *ntdAa-lacZ* fusion in *Burkholderia cepacia* JS872 after growth in the presence of various potential inducing and repressing compounds. Sal, salicylate; succ, succinate; glc, glucose.

Purification and characterization of NBDO and 2NTDO components. The protein components of the 2NTDO and NBDO enzyme systems from *Acidovorax* sp. strain JS42 and *Comamonas* sp. strain JS765, respectively, were purified and characterized (Table IV-4; Figure IV-14). The identical shared reductase and ferredoxin components were monomers of 35 and 11.5 kD, respectively. The reductase component contained 1.86 g-atoms iron, 2.01 g-atoms sulfur and one molecule of FAD per monomer. Spectral properties of the reductase indicated the presence of a plant-type [2Fe-2S] center and a flavin. The ferredoxin contained 2.20 g-atoms iron and 1.99 g-atoms sulfur per monomer and had spectral properties indicative of a Rieske [2Fe-2S] center. The ferredoxin component could be effectively replaced by the ferredoxin from the *Pseudomonas* sp. NCIB 9816-4 naphthalene dioxygenase system, but not those from the *Burkholderia* sp. LB400 biphenyl or *Pseudomonas putida* F1 toluene dioxygenase systems. The oxygenases from the 2NTDO and NBDO systems each had spectral properties indicating the presence of a Rieske [2Fe-2S] center, and the subunit composition of each oxygenase was an $\alpha_3\beta_3$ hexamer. The apparent K_m of 2-nitrotoluene dioxygenase for 2NT was 20 μM, and that for naphthalene was 121 μM. The specificity constants were 7.0 μM^{-1} min^{-1} for 2NT and 1.2 μM^{-1} min^{-1} for naphthalene, indicating that the enzyme is more efficient with 2NT as a substrate. Details of this study have been published (22).

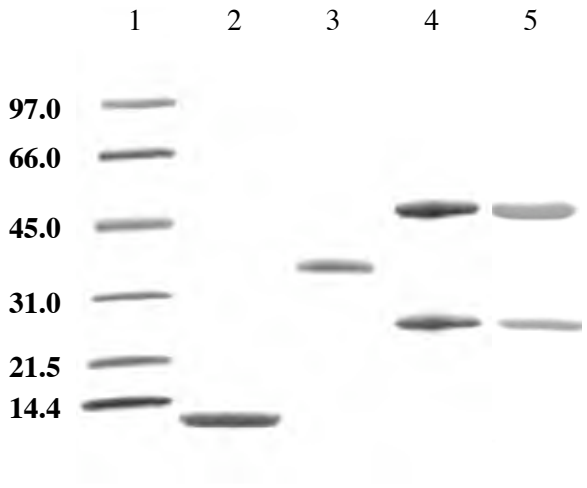


Figure IV-14. SDS-PAGE analysis of purified 2NTDO and NBDO components. Lane 2, $Ferredoxin_{2NT}$, Lane 3, $Reductase_{2NT}$, Lane 4, $Oxygenase_{2NT}$, Lane 5, $Oxygenase_{NBZ}$. Each lane was loaded with 5 µg of protein. Lane 1, molecular weight standards. Protein was stained with Coomassie brilliant blue R250.

Table IV-4. Summary of physical properties of 2NTDO and NBDO protein components.

Properties	$Reductase_{2NT}$	$Ferredoxin_{2NT}$	$Oxygenase_{2NT}$	$Oxygenase_{NBZ}$
Molecular Mass, kDa	36.2[a]	11.5[a]	210[a]	216[b]
Subunit molecular mass, kDa[c]	35.8, (35.303)	10.5, (11.481)	□ 50.4, (49.490)	□ 51.2, (49.561)
(from deduced amino acid sequence)[d]			□ 25.5, (23.091)	□ 25.3, (23.088)
Subunit structure	monomer	monomer	heterotrimer	heterotrimer
Iron (g-atoms per mol)[e]	1.86 ± 0.07	2.20 ± 0.30	2.85 ± 0.08/□□	2.25 ± 0.17/□□
Acid labile sulfur, g-atoms per mol[e]	2.01 ± 0.20	1.99 ± 0.02	1.91 ± 0.19/□□	2.11 ± 0.09/□□
FAD, mol per mol[f]	1.01 ± 0.17; 1.06 ± 0.02	-	-	-
Absorption spectra, $\lambda_{max,nm}$	271, 344, 398, 460	326, 456	326, 454	330, 460
Extinction coeff., $mM^{-1}cm^{-1}$ ($\lambda_{max,nm}$)[e]	20.8 (398), 22.5 (460)	11.7 (326), 5.8 (456)	17.8/□□ (326), 6.5/□□ (454) 6.6/□□ (460)	11.9/□□ (330),
EPR (reduced), g_x g_y g_z	2.03, 2.003, 1.93	2.02, 1.90, 1.81	2.01, 1.91, 1.78	2.02, 1.91, 1.75
Gene designation	*ntdAa*	*ntdAb*	*ntdAc,Ad*	*nbzAc,Ad*
N-terminal amino acid sequence	MELWEP-	SENWIDAIAR- □ MMINTQEDKLVS-	□ SYQNLVSEAGLT- □ MMINTQEDKLV-	□ SYQNLVSEAG-

[a] Determined by gel filtration;
[b] Determined by dynamic light scattering;
[c] Determined by SDS-PAGE;
[d] Data from (14, 19).
[e] Determined as described in Materials and Methods, standard deviations are shown; [f] Determined by HPLC and spectroscopy, respectively (see Materials and Methods), standard deviations are shown. The molecular mass determined from the deduced amino acid sequence (24, 29) was used in all calculations.

Substrate specificity of NBDO and 2NTDO. The substrate specificity of 2NTDO was previously reported (20). A number of substituted aromatic compounds were hydroxylated by NBDO (Table IV-5). The results show that a nitro-substituted position was generally the preferred, but not exclusive, oxidation site as shown by production of nitrobenzyl alcohol isomers from 2NT and 4NT, dinitrobenzyl alcohol from 2,4-DNT, and the nitro-substituted dihydrodiols from 2-chloro-4-nitrotoluene and 1-nitronaphthalene.

Transformation of 1-nitronaphthalene by NBDO yielded two hydroxylated products. A small amount of 1,2-dihydroxynaphthalene was formed with the concomitant release of nitrite. The amount of 1,2-dihydroxynaphthalene detected by LC or GC analysis did not correspond well with the concentration of nitrite measured. The discrepancy is probably due to the known instability of the 1,2-dihydroxynaphthalene. The major product formed from 1-nitronaphthalene gave a mass spectrum consistent with a nitronaphthalene *cis*-dihydrodiol. Only one compound gave the appropriate molecular ion for a nitro-dihydrodiol, indicating a highly regiospecific reaction with 1-nitronaphthalene. In fact, mixtures of dihydroxylated products were identified with all three of the latter substrates. The mixtures indicate an imprecise fit of those substrates and the NBDO.

Naphthalene and toluene were tested as substrates to determine the specificity of NBDO with non-nitro containing aromatic compounds. Naphthalene was transformed to naphthalene *cis*-1,2-dihydrodiol, the same product formed by naphthalene dioxygenase. However, the diol formed by NBDO was not homochiral. The enantiomeric composition of this product was 57% with respect to the (+)-(1*R*,2*S*)-enantiomer. NBDO transformed toluene to toluene *cis*-2,3-dihydrodiol, in contrast to NDO which oxidizes toluene to benzyl alcohol.

Table IV-5. Products formed from aromatic substrates by NBDO in DH5(pDTG927)

Substrate	Product(s)[a]	Retention time (min)	Relative yield %
Nitrobenzene	**Catechol**	5.5	100
2-Nitrotoluene	**3-Methylcatechol**	6.5	55[b]
	2-Nitrobenzyl alcohol	7.6	45[b]
3-Nitrotoluene	**4-Methylcatechol**	6.4	100
4-Nitrotoluene	**4-Methylcatechol**	6.4	85
	4-Nitrobenzylalcohol	8.8	15
Naphthalene	**Naphthalene *cis*-1,2-dihydrodiol**	9.6, 10.6[c]	100
2,3-Dinitrotoluene	4-Methyl-3-nitrocatechol	9.9	100
2,4-Dinitrotoluene	4-Methyl-3-nitrocatechol	9.9	21
	4-Methyl-5-nitrocatechol	10.9	62
	2,4-Dinitrobenzylalcohol	11.0	17
2,6-Dinitrotoluene	**3-Methyl-4-nitrocatechol**	11.4	100
3,4-Dinitrotoluene	4-Methyl-6-nitrocatechol	10.4	100
1,3-Dinitrobenzene	**4-Nitrocatechol**	10.5	100
1,4-Dinitrobenzene	**4-Nitrocatechol**	8.4	100
2-Chloro-4-nitrotoluene	3-Chloro-4-methylcatechol	8.4	68
	4-Chloro-5-methylcatechol	8.5	19
	4-Chloro-3-methyl-6-nitrophenol[d]	9.5	7
	3-Chloro-2-methyl-5-nitrophenol[d]	9.8	6
2-Chloro-6-nitrotoluene	4-Chloro-3-methylcatechol	8.8	100
1-Nitronaphthalene	1,2-Dihydroxynaphthalene	10.9, 11.4[c]	1
	cis-(1,2)-Dihydroxy-1,2-dihydro-8-nitronaphthalene	12.8, 14.4[c]	99
Toluene	**Toluene *cis*-2,3-dihydrodiol**	5.8, 5.9[c]	100

[a] Product identity based on GC-MS analysis of TMS derivatives compared with standards (bold) or interpretation of GC-MS, LC, and absorption spectral data (normal type face).
[b] Relative yield of products formed from 2NT were determined without derivatization.
[c] Derivatized with butylboronate.
[d] Phenols formed from dehydration of putative unstable *cis*-2,3-dihydro-6-chloro-4-nitrotoluenediol.

The reaction rates found for the NBDO with various substrates also indicated a relatively relaxed substrate preference. Purified oxygenase proteins were combined to serve as catalyst in assays that tested reaction rates with several aromatic substrates. The reaction rates with the three mononitrotoluene isomers gave further insight into the catalytic specificity of the enzyme (Table IV-6). Both 2NT and 4NT yielded mixtures of oxidation products, although the monohydroxylated product had a much higher relative rate of formation when 2NT was provided as the substrate. The experiments with dinitrobenzene isomers demonstrated clear differences in substrate preference. Oxidation of both the 1,3- and 1,4-isomers resulted in the production of 4-nitrocatechol. However, the preference for oxygenolytic elimination of *meta*-nitro substituents was obvious from the comparison of oxidation rates with 1,3-dinitrobenzene and 1,4-dinitrobenzene as substrates. Oxidation of 1,4-dinitrobenzene was not measurable in the assays with reconstituted enzyme, while the rate of 4-nitrocatechol formation from 1,3-dinitrobenzene was comparable to the transformation rate with nitrobenzene. Details of this study have been published (14).

Table IV-6. Substrate specificity of NBDO

Substrate	Products	Relative Activity[a]
Nitrobenzene	Catechol + NO_2^-	100
2-Nitrotoluene	3-Methylcatechol + NO_2^-	59
	2-Nitrobenzylalcohol	67
3-Nitrotoluene	4-Methylcatechol + NO_2^-	221
4-Nitrotoluene	4-Methylcatechol + NO_2^-	42
	4-Nitrobenzylalcohol	3
1,3-Dinitrobenzene	4-Nitrocatechol + NO_2^-	89
1,4-Dinitrobenzene	[4-Nitrocatechol + NO_2^-][b]	bd[c]
2,6-Dinitrotoluene	3-Methyl-4-nitrocatechol + NO_2^-	24
1-Nitronaphthalene	1,2-Dihydroxynaphthalene + NO_2^-	30
Naphthalene	Naphthalene *cis*-1,2-dihydrodiol	59

[a] Reactions (final volume 750 µl) contained 100 µM substrate, 200 µM NADH, 19 µg $Reductase_{2NT}$, 31 µg $Ferredoxin_{2NT}$, and 52 µg $Oxygenase_{NBZ}$ in 50 mM MES buffer, pH 6.8. Samples were collected after two minutes incubation. The amounts of nitrite evolved and hydroxylated transformation products that accumulated following incubation were determined as described in Materials and Methods. The values reported are the averages of duplicate reactions with respect to the specific activity with nitrobenzene as the substrate (302 nmol min^{-1} (mg of $Oxygenase_{NBZ}$)$^{-1}$).

[b] No products were detected in this reconstituted enzyme assay. Products listed were identified in the whole-cell biotransformation assays (Table 2).

[c] Below detection. (< 5 nmol min^{-1} (mg of $Oxygenase_{NBZ}$)$^{-1}$).

Analysis of mutant forms of 2NTDO an NBDO. Naphthalene dioxygenase from strain NCIB 9816-4 has relaxed substrate specificity and is able to oxidize more than 70 substrates (24). However, it is interesting that naphthalene dioxygenase is unable to dihydroxylate nitroaromatic compounds at the nitro-substituted carbon, despite the identity shared with nitroarene dioxygenases. In fact, the substrate oxidation profiles for naphthalene dioxygenase, NBDO, 2NTDO, and 2,4-DNTDO are very different. Thus, the amino acid residues that are unique to the active sites in the oxygenase □-subunit most likely account for the enzyme specificity differences between NBDO, 2NTDO, 2,4-DNTDO, and naphthalene dioxygenase. To identify the specific residues at the active sites of NBDO and 2NTDO that confer the ability to oxidize nitroarene compounds with release of nitrite, we initiated a study of the active sites of the enzymes.

By generating and analyzing mutant forms of the enzyme, we identified specific amino acids at the active site of 2NTDO that control specificity. The enzymes were tested for the ability to oxidize mono- and dinitrotoluenes, as well as ADNTs. The residue at position 350 was found to be critical in controlling the ability to oxidize the ring of mononitrotoluenes (Table IV-7). Substitution of Ile350 by phenylalanine resulted a severely reduced the ability of the enzyme to produce methylcatechols from nitrotoluenes. Instead, the methyl group of each nitrotoluene isomer was preferentially oxidized to form the corresponding nitrobenzyl alcohol. Substitution of a valine at position 258 significantly changed ability of the enzyme to oxidize the aromatic ring of nitrotoluenes. None of the mutant forms of the enzyme had improved activity with DNTs or ANDTs. Based on active site modeling using the crystal structure of NBDO from *Comamonas* sp. JS765 (6), Asn258 appears to contribute to substrate specificity through hydrogen bonding to the nitro group of mononitrotoluenes. A manuscript describing this work is in press (13).

We used site-directed mutagenesis to identify residues at the active site of NBDO that contribute to substrate specificity. The activities of six mutant forms of NBDO were tested with nitrobenzene, mono- and dinitrotoluenes, and ADNTs. Results indicated that the residues at position 258, 293, and 350 in the □ subunit play important roles in determining regiospecificity with nitroarene substrates (Figures IV-15-18). The results also provide an explanation for the characteristic specificity with mononitrotoluene substrates. Based on the structure of NBDO (6), substitution of a valine for the asparagine at position 258 eliminated an essential hydrogen bond between the substrate nitro group and the amino group of asparagine. Similarly, substitution of a large residue (phenylalanine) for the isoleucine at position 350 appeared to prevent formation of the hydrogen bond by steric interference. In both cases, oxidation of mononitrotoluenes by these mutant enzymes was predominantly at the methyl group rather than on the aromatic ring. Specificity of enzymes with substitutions at position 293 varied depending on the amino acid present. None of the enzymes were improved in ADNT oxidation, but one mutant enzyme (F293Q) was significantly more efficient at oxidizing 2,6-DNT than the wild type based on product formation rates and whole cell kinetics (Table IV-8). This enzyme may prove useful for pathway engineering. A manuscript describing this work has been submitted (10).

Table IV-7. Substrate specificity of mutant forms of 2NTDO with mononitrotoluenes

Substrate[b]	Metabolite[c]	Products[a] (%) formed by 2NTDO enzymes:					
		Wild type	N258V	W314F	N258V/ W314F	I350F	I350T
2NT							
	3-MC	84	-[d]	80	1	7	72
	2-NBA	16	>99	20	99	93	28
3NT							
	3-MC	14	3	49	7	3	50
	4-MC	60	16	24	9	10	20
	3-NBA	26	81	27	84	87	30
4NT							
	4-MC	97	82	88	31	3	15
	4-NBA	3	18	12	69	97	85

[a] Product ratios were determined from integration of the GC-MS total ion current chromatograms. Results reported are the averages of at least three independent experiments, and standard deviations were less than 10%.
[b] NT, nitrotoluene.
[c] MC, methylcatechol; NBA nitrobenzyl alcohol.
[d] - not detected.

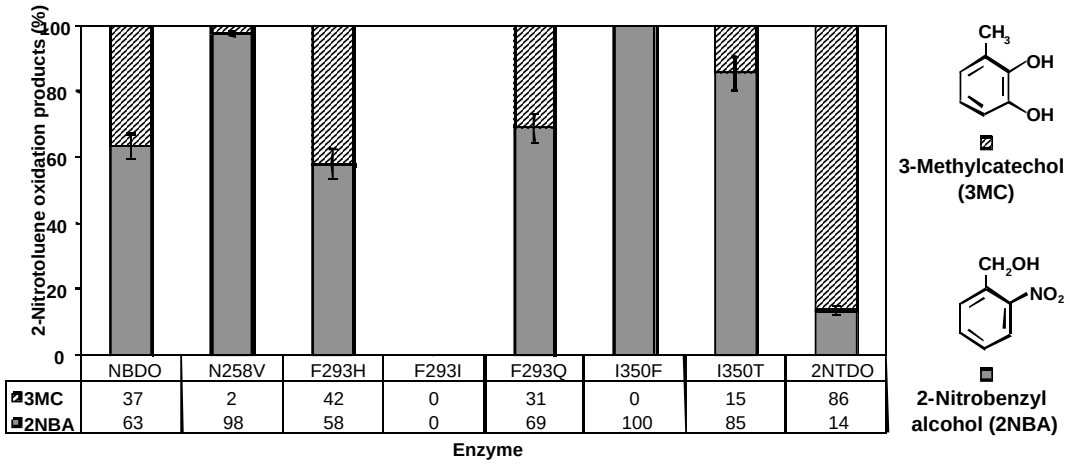


Figure IV-15. Oxidation products from 2NT biotransformation reactions with wild-type NBDO, NBDO variants, and 2NTDO (n = 3). 3-Methylcatechol (3MC) is represented in stippled white bars and 2-nitrobenzyl alcohol (2NBA) in dark gray bars.

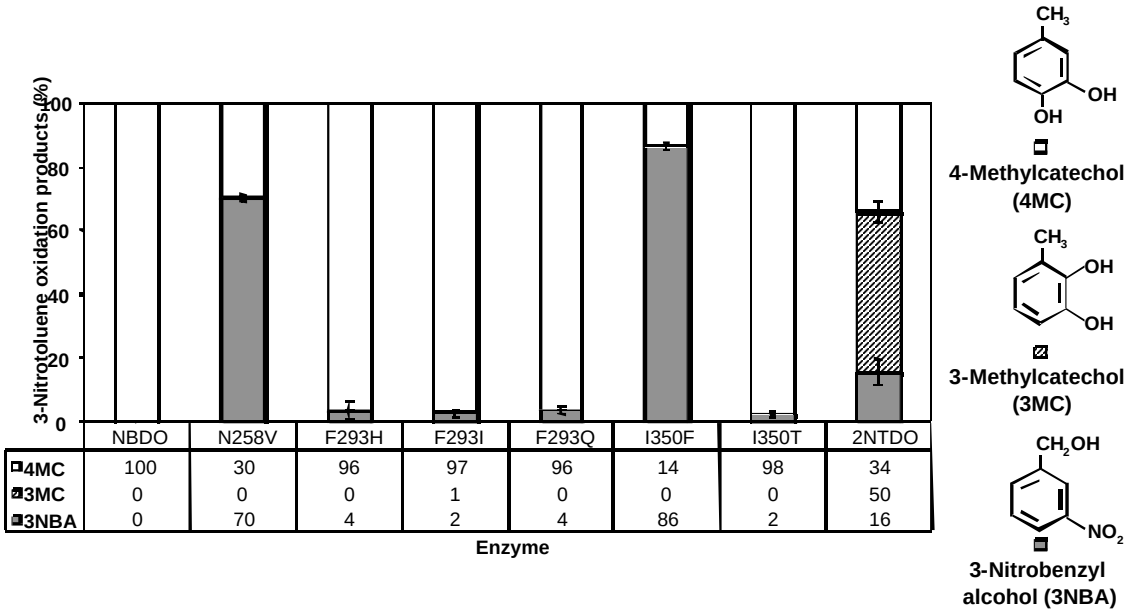


Figure IV-16. Oxidation products from 3NT biotransformation reactions with wild-type NBDO, NBDO variants, and 2NTDO (n = 3). 4-Methylcatechol (4MC) is represented in white bars, 3-methylcatechol (3MC) in stippled white bars and 3-nitrobenzyl alcohol (3NBA) in dark gray bars.

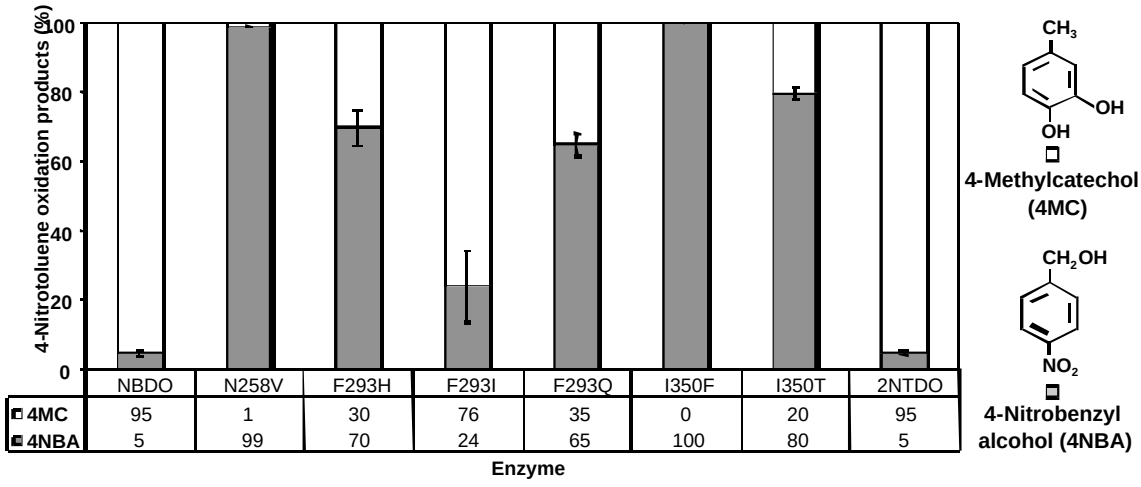


Figure IV-17. Oxidation products from 4NT biotransformation reactions with wild-type NBDO, NBDO variants, and 2NTDO (n = 3). 4-Methylcatechol (4MC) is represented in white bars and 4-nitrobenzyl alcohol (4NBA) in dark gray bars.

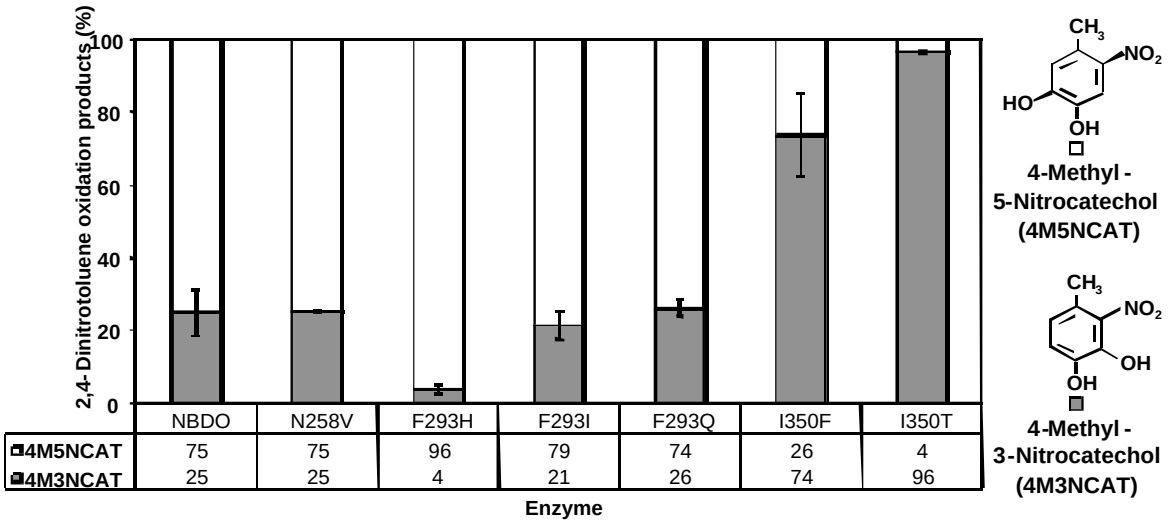


Figure IV-18. Oxidation products from 24-DNT biotransformation reactions (n = 3) with wild-type NBDO, NBDO variants, and 2NTDO. 4-Methyl-5-nitrocatechol (4M5NCAT) is represented in white bars and 4-methyl-3-nitrocatechol (4M3NCAT) in dark gray bars.

Table IV-8. Nitrobenzene and 2,6-DNT as substrates:
Nitrite formation and whole cell biotransformation kinetics

Enzyme	Mean nitrite released[a] ± SD from:		Estimated kinetic coefficients[b]			
	Nitrobenzene	2,6-DNT	Nitrobenzene		2,6-DNT	
			K_m (μM)	V_{max}	K_m (μM)	V_{max}
NBDO	923 ± 55	563 ± 77	7.7 ± 0.2	3.6 ± 0.1	851 ± 131	5.48 ± 1.25
-N258V	81.6 ± 3.1	384 ± 43	1780 ± 70	0.23 ± 0.12	808 ± 69	3.20 ± 2.74
-F293H	127 ± 44	281 ± 128	23.9 ± 1.4	2.0 ± 1.4	12.0 ± 4.2	0.51 ± 0.21
-F293I	39.8 ± 16.3	25.8 ± 17.5	ND[c]	BLD[d]	288 ± 55	1.74 ± 1.29
-F293Q	1080 ± 180	2040 ± 320	60.7 ± 12.8	4.5 ± 2.0	717 ± 44	13.5 ± 2.0
-I350F	41.9 ± 5.6	7.7 ± 3.9	ND	BLD	ND	BLD
-I350T	1380 ± 290	43.1 ± 23.8	159 ± 35	16 ± 1	ND	2.83 ± 0.42[e]
2NTDO	1870 ±110	7.2 ± 3.0	21.0 ± 4.1	12.1 ± 1.7	ND	0.56 ± 0.25[e]

[a] Expressed as nmol NO_2^- mg protein^{-1}. Nitrobenzene and 2,6-DNT products were assayed after 6 and 16 h, respectively.
[b] Kinetic coefficients were estimated for whole cells expressing dioxygenase variants by non-linear regression with the Michaelis-Menten equation (31). V_{max} is reported as nmol min^{-1} mg protein^{-1}.
[c] ND, could not be determined. These reactions were very slow and did not follow Michaelis-Menten kinetics.
[d] BLD, below the limit of detection.
[e] Reactions did not follow Michaelis-Menten kinetics. Reported values reflect maximum observed oxidation rate under substrate saturating conditions (1 mM).

Conclusions

Nitroarene dioxygenases have been shown to have the unique ability to oxidize specific subsets of nitroarene substrates at the nitro-substituted carbon, reactions not catalyzed by related aromatic hydrocarbon dioxygenases such as naphthalene dioxygenase (NDO). The basis for this ability was not clear prior to this study. In the case of NBDO and 2NTDO, it appears that correct positioning of nitrobenzene and mononitrotoluene substrates in the active site is dependent on the presence of an asparagine residue at position 258, which forms a hydrogen bond to the nitro group of the substrate. In the absence of this residue, either in mutant forms of the enzyme or in related enzymes such as DNTDO and NDO, oxidation does not occur at the nitro-substituted carbon. DNTDOs, which do not catalyze mononitrotoluene oxidation with nitrite release, do not have an asparagine at position 258. Instead, the residue at position 293 may be important in positioning DNTs in the appropriate location in the active site for ring oxidation and nitrite removal. Results of this study have shown NBDO to be a very versatile enzyme that is capable of oxidation and nitrite removal from nitrobenzene, 2-, 3-, and 4NT, 2,4- and 2,6-DNT, and 4ADNT. One mutant form of NBDO (F293Q) was also found to have improved activity with 2,6-DNT.

The results presented here demonstrate that the genes encoding the nitroarene dioxygenases in strains JS765 and JS42 are located in inducible operons, and that each requires a LysR-type regulator for expression. Each operon closely parallels the *nah* and *sal* operons for naphthalene and salicylate degradation in *P. putida* G7. The regulatory proteins are similar, inducers of the *nah/sal* operons induce the nitroarene operons, and the *nahR* binding site is present. Together with overall sequence similarities and enzyme activities, these results strongly suggest that the nitroarene dioxygenase gene clusters evolved from ancestral naphthalene gene clusters. However, the *ntd* and *nbz* genes are inducible in the presence of nitrobenzene, mononitrotoluenes, DNTs, ADNTs and even TNT, indicating that the genes will be efficiently expressed in environments contaminated with mixtures of nitroarene compounds. The results also show that within a very short evolutionary time period, these nitroarene dioxygenase operons have evolved appropriate regulatory systems for the control of the degradation genes. The regulation of the DNTDO genes appears to be different, and is more similar to that of the naphthalene catabolic operons. DntR and NagR are capable of responding to salicylate, but not nitroarene inducers. We are currently examining the basis for the inducer specificities of NtdR and NagR by generating and characterizing mutant and hybrid regulatory proteins.

Although NtdR supports a higher uninduced level of expression in JS42 and responds to nitroarene inducers in JS42 and JS765 strain backgrounds, we have been unable to move NtdR to other strains and show induction by nitroarene compounds. We are still working to understand this phenomenon, but at this point, the use of the *ntd* promoter-regulator pair to control expression of an engineered pathway in an alternative host strain is not feasible (go/no go). One possible alternative is to use JS42 or JS765 as the host, but these strains are not as robust as, for example, pseudomonads, and we typically need to supplement culture medium with yeast extract or vitamins to get good growth. Whether engineered forms of these strains would be competitive in the environment remains to be tested.

Literature Cited

1. **An, D., D. T. Gibson, and J. C. Spain.** 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. J. Bacteriol. **176:**7462-7467.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.
3. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568-6572.
4. **Dunn, N. W., and I. C. Gunsalus.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. **114:**974-979.
5. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. Gene **52:**147-54.
6. **Friemann, R., M. M. Ivkovic-Jensen, D. J. Lessner, C.-L. Yu, D. T. Gibson, R. E. Parales, H. Eklund, and S. Ramaswamy.** 2005. Structural insights into the dioxygenation of nitroarene compounds: The crystal structure of the nitrobenzene dioxygenase. J. Mol. Biol. **348:**1139-1151.
7. **Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.).** 1994. Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
8. **Haigler, B. E., W. H. Wallace, and J. C. Spain.** 1994. Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42. Appl. Environ. Microbiol. **60:**3466-3469.
9. **Jones, R. M., B. Britt-Compton, and P. A. Williams.** 2003. The naphthalene catabolic (*nag*) genes of *Ralstonia* sp. strain U2 are an operon that is regulated by NagR, a LysR-type transcriptional regulator. J. Bacteriol. **185:**5847-5853.
10. **Ju, K.-S., and R. E. Parales.** 2005. Control of substrate specificity by active site residues in nitrobenzene 1,2-dioxygenase. Appl. Environ. Microbiol. **Submitted**.
11. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191-197.
12. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad host range cloning vector. BioTechniques **16:**800-802.
13. **Lee, K.-S., J. V. Parales, R. Friemann, and R. E. Parales.** 2005. Active site residues controlling substrate specificity in 2-nitrotoluene dioxygenase from *Acidovorax* sp. strain JS42. J. Ind. Microbiol. Biotechnol. **In press**.
14. **Lessner, D. J., G. R. Johnson, R. E. Parales, J. C. Spain, and D. T. Gibson.** 2002. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. **68:**634-641.
15. **Lessner, D. J., R. E. Parales, S. Narayan, and D. T. Gibson.** 2003. Expression of nitroarene dioxygenase genes in *Comamonas* sp. strain JS765 and *Acidovorax* sp. strain JS42 is induced by multiple aromatic compounds. J. Bacteriol. **185:**3895-3904.
16. **Miller, J. H.** 1975. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor , N.Y.

17. **Nishino, S. F., G. C. Paoli, and J. C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and the pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66:**2139-2147.
18. **Nishino, S. F., and J. C. Spain.** 1995. Oxidative pathway for the biodegradation of nitrobenzene by *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. **61:**2308-2313.
19. **Parales, J. V., A. Kumar, R. E. Parales, and D. T. Gibson.** 1996. Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. Gene **181:**57-61.
20. **Parales, J. V., R. E. Parales, S. M. Resnick, and D. T. Gibson.** 1998. Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the C-terminal region of the □ subunit of the oxygenase component. J. Bacteriol. **180:**1194-1199.
21. **Parales, R. E., M. D. Emig, N. A. Lynch, and D. T. Gibson.** 1998. Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems. J. Bacteriol. **180:**2337-2344.
22. **Parales, R. E., R. Huang, C.-L. Yu, J. V. Parales, F. K. N. Lee, M. M. Ivkovic-Jensen, W. Liu, D. J. Lessner, R. Friemann, S. Ramaswamy, and D. T. Gibson.** 2005. Purification, characterization, and crystallization of the components of the nitrobenzene and 2-nitrotoluene dioxygenase enzyme systems. Appl. Environ. Microbiol. **71:**3806-3814.
23. **Parales, R. E., T. A. Ontl, and D. T. Gibson.** 1997. Cloning and sequence analysis of a catechol 2,3-dioxygenase gene from the nitrobenzene-degrading strain *Comamonas* sp. JS765. J. Ind. Microbiol. Biotechnol. **19:**385-391.
24. **Resnick, S. M., K. Lee, and D. T. Gibson.** 1996. Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J. Ind. Microbiol. **17:**438-457.
25. **Resnick, S. M., D. S. Torok, K. Lee, J. M. Brand, and D. T. Gibson.** 1994. Regiospecific and stereoselective hydroxylation of 1-indanone and 2-indanone by naphthalene dioxygenase and toluene dioxygenase. Appl. Environ. Microbiol. **60:**3323-3328.
26. **Sambrook, J., E. F. Fritch, and T. Maniatis.** 1989. Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
27. **Schell, M. A.** 1983. Cloning and expression in *Escherichia coli* of the naphthalene degradation genes from plasmid NAH7. J. Bacteriol. **153:**822.
28. **Schell, M. A.** 1990. Regulation of naphthalene degradation genes of plasmid NAH7: example of a generalized positive control system in *Pseudomonas* and related bacteria, p. 165-177. *In* A. M. Chakrabarty, S. Kaplan, B. Iglewski, and S. Silver (ed.), Pseudomonas: Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
29. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784-789.
30. **Smirnova, I. A., C. Dian, G. A. Leonard, S. McSweeney, D. Birse, and P. Brzezinski.** 2004. Development of a bacterial biosensor for nitrotoluenes: the crystal structure of the transcriptional regulator DntR. J. Mol. Biol. **340:**405-418.

31. **Smith, L. H., P. L. McCarty, and P. K. Kitanidis.** 1998. Spreadsheet method for evaluation of biochemical reaction rate coefficients and their uncertainties by weighted nonlinear least-squares analysis of the integrated monod equation. Appl. Environ. Microbiol. **64:**2044-2050.
32. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159-271.
33. **Suen, W.-C., B. E. Haigler, and J. C. Spain.** 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderi*a sp. strain DNT: Similarity to naphthalene dioxygenase. J. Bacteriol. **178:**4926-4934.
34. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074-1078.
35. **Whiteley, M., and E. P. Greenberg.** 2001. Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. J. Bacteriol. **183:**5529-5534.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.
37. **Zhou, N.-Y., J. Al-Dulayymi, M. S. Baird, and P. A. Williams.** 2002. Salicylate 5-hydroxylase from *Ralstonia* sp. strain U2: a monooxygenase with close relationships to and shared electron transport proteins with naphthalene dioxygenase. J. Bacteriol. **184:**1547-1555.
38. **Zhou, N.-Y., S. L. Fuenmayor, and P. A. Williams.** 2001. *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. J. Bacteriol. **183:**700-708.

V. Oxidative transformation of aminodinitrotoluene isomers by multicomponent oxygenases

Background

Bacterial degradation of aromatic compounds under aerobic conditions typically begins with dihydroxylation of the aromatic ring. The reaction can be catalyzed by multicomponent dioxygenases, which incorporate both atoms of an oxygen molecule via an electrophilic attack on the ring. Oxygenase-catalyzed hydroxylation of TNT is therefore hampered by the three nitro groups depriving the ring of electrons (3).

The ability to reduce nitro groups on aromatic rings is common among organisms because the polarized N—O bond makes the group readily reducible (1). A number of enzymes will catalyze gratuitous reductions of TNT (10) and the typically observed biological transformation of TNT under aerobic conditions is a series of 2 electron transfers to form the corresponding; nitroso, hydroxylamino, and amino forms of the original nitroarene (Figure V-1). Reports have repeatedly described aerobic transformations of TNT that yield aminodinitrotoluene (ADNT) isomers as products (4-9). The electron deficiency of the ADNTs is less pronounced than that of TNT, suggesting greater susceptibility to oxidative degradation. Our experiments tested whether nitroarene dioxygenases from strains that oxidatively degrade 2,4-DNT or nitrobenzene would transform ADNT isomers.

Results

Three nitroarene dioxygenases were tested for ADNT transformation. Recombinant strains carrying the dioxygenase genes were incubated with 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene; substrate consumption and product accumulation were determined using HPLC and standard chemical methods. Aromatic products were identified using gas chromatography and mass spectroscopy.

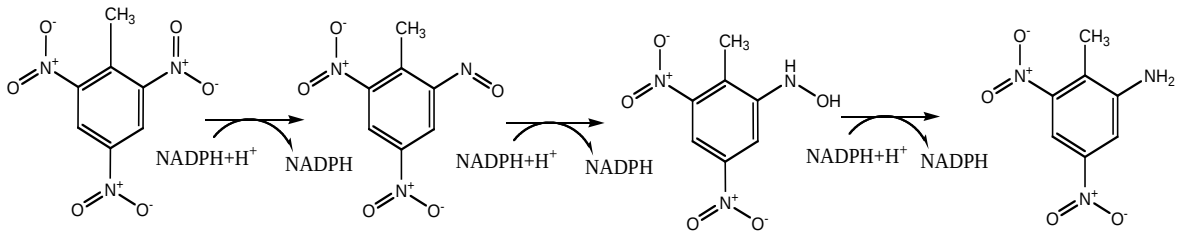


Figure V-1. Enzymatic reduction of TNT to 2-amino-4,6, dinitrotoluene. 4-Amino-2,6-dinitrotoluene can be formed in equivalent reactions, with reduction at the nitro group that is para-to the methyl substituent.

The three nitroarene dioxygenases show clear distinctions in catalytic specificity (Figure V-2). The nitrobenzene dioxygenase (NBDO) from *Comamonas* sp. strain JS765 transformed both ADNT isomers. 2-ADNT was attacked at the methyl-substituent to yield 2-amino-4,6-

dinitrobenzylalcohol. With 4-ADNT as the substrate, the nitrobenzene dioxygenase catalyzed the dioxygenation of the aromatic ring to yield nitrite and 3-amino-6-methyl-5-nitrocatechol. The 2,4-dinitrotoluene dioxygenases from *Burkholderia* sp. strain DNT and *Burkholderia cepacia* R34 were also evaluated. Neither of the 2,4-DNT dioxygenases(24DNTDO) attacked 4ADNT, however, both transformed 2ADNT. The dioxygenase from strain DNT catalyzed dioxygenation of the ring to yield nitrite and a catechol, 3-amino-4-methyl-5-nitrocatechol. The dioxygenase from strain R34 demonstrated less catalytic specificity, both 3-amino-4-methyl-5-nitrocatechol and 2-amino-4,6-dinitrobenzylalcohol were isolated from the reaction supernatant. None of the oxygenases attacked the methyl group of 4-amino-2,6-dinitrotoluene. The results of this study have been reported (2).

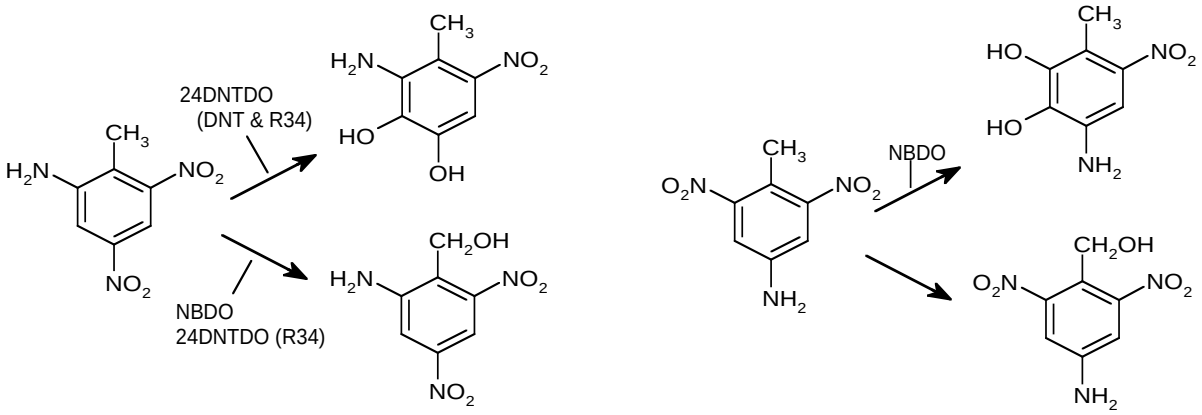


Figure V-2. Summary of ADNT hydroxylation by the nitroarene dioxygenases (2).

Conclusions

The specific dihydroxylation of ADNT isomers has potential practical significance for biological degradation of TNT. The oxidative bacterial transformation of ADNT under aerobic conditions might account for previously observed losses of TNT during aerobic treatment of mixtures of nitroaromatic compounds. It is known that TNT is gratuitously reduced to ADNTs in the environment (4-9), the findings may provide a useful alternative (indirect) route to TNT oxidation. Moreover, removal of another electron-withdrawing nitro substituent from the ring via the catechol formation, should favor additional microbial transformations.

Literature Cited

1. **Esteve-Nuñez, A., A. Caballero, and J. L. Ramos.** 2001. Biological degradation of 2,4,6-trinitrotoluene. Microbiol Mol Biol Rev **65:**335-352.
2. **Johnson, G. R., B. F. Smets, and J. C. Spain.** 2001. Oxidative transformation of amindinitrotoluene isomers by multicomponent dioxygenases. Appl. Environ. Microbiol. **67:**5460-5466.
3. **Lenke, H., C. Achtnich, and H.-J. Knackmuss.** 2000. Perspectives of bioelimination of polynitroaromatic compounds, p. 91-126. *In* J. C. Spain, J. B. Hughes, and H.-J.

Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. Lewis, Boca Raton, Florida.

4. **Lewis, T. A., M. M. Ederer, R. L. Crawford, and D. L. Crawford.** 1997. Microbial transformation of 2,4,6-trinitrotoluene. Ind. Microbiol. Biotechnol. **18:**89-96.
5. **Lewis, T. A., D. A. Newcombe, and R. L. Crawford.** 2004. Bioremediation of soils contaminated with explosives. Journal of Environmental Management **70:**291-307.
6. **McCormick, N. G., F. E. Feeherry, and H. S. Levinson.** 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Microbiol. **31:**949-958.
7. **Naumova, R. P., N. N. Amerkhánova, and L. N. Zolotukhina.** 1984. Peculiarities of nitroreduction as a key stage in the microbial destruction of aromatic nitrocompounds. Applied Biochemistry and Microbiology **19:**400-404.
8. **Oh, B.-T., G. Sarath, and P. J. Shea.** 2001. TNT nitroreductase from a *Pseudomonas aeruginosa* strain isolated from TNT-contaminated soil. Soil Biology and Biochemistry **33:**875-881.
9. **Oh, B.-T., P. J. Shea, R. A. Drijber, G. K. Vasilyeva, and G. Sarath.** 2003. TNT biotransformation and detoxification by a *Pseudomonas aeruginosa* strain. Biodegradation **14:**309-319.
10. **Riefler, R. G., and B. F. Smets.** 2000. Enzymatic reduction of 2,4,6-trinitrotoluene and related nitroarenes: kinetics linked to one-electron redox potentials. Environ. Sci. Technol. **34:**3900-3906.

VI. Characterization of nitroreductase activity in potential host strains

Summary

In our strategy to generate an engineered TNT-degradation pathway, one of the required steps involves the reduction of TNT to ADNT. Many bacterial strains are capable of carrying out this reaction, but others generate reactive hydroxyaminodinitrotoluenes (HADNTs). In order to identify a useful host strain to carry the engineered pathway, we screened the native nitroreductase activities present in two strains of *E. coli*, and two *Pseudomonas* strains. In addition, we tested the contribution of two previously studied nitroreductases (NfsA and NfsB) from *E. coli* in order to determine whether introduction of these genes would improve the conversion of TNT to ADNT. Our results demonstrated that all strains produced 4-amino-2,6-DNT as the major TNT reduction product. The *E. coli* strains were also capable of producing significant amounts of HADNTs and diaminonitrotoluenes (DANTs). The *Pseudomonas* strains produced predominantly 4-amino-2,6-DNT, but also made some 2-amino-4,6-DNT. However, HADNTs and DANT were not generated. A definitive role for *nfsA* and *nfsB* could not be determined, but introduction of either gene did not improve ADNT production in any of the tested strain backgrounds. Based on our results, either *P. putida* KT2440 or *Pseudomonas* sp. strain Idaho may be a good host strain to harbor a TNT pathway that proceeds through 4ADNT.

Background

Although dioxygenase enzymes capable of oxidizing nitrobenzene, 2NT, 2,4-DNT, and 2,6-DNT have been identified, these enzymes have no detectable activity with TNT. In addition, none of the mutant dioxygenase enzymes that we have generated during the course of this study were capable of oxidizing TNT (go/no go). However, we have demonstrated that several nitroarene dioxygenases were capable of aminodinitrotoluene (ADNT) oxidation (13). Therefore, early in our studies, we modified our planned TNT degradation strategy to include an initial reduction step, converting TNT to ADNT. Reduction of nitro groups is a nonspecific activity catalyzed by nitroreductases present in a wide range of bacterial species (2, 7, 18, 19). The ability to reduce nitro groups on aromatic rings is common because the polarized nature of the N—O bond makes the group readily reducible (8). The typically observed biological transformation of TNT under aerobic conditions is a series of 2 electron transfers to form the corresponding; nitroso, hydroxylamino, and amino forms of the original nitroarene. The reduction is referred to as oxygen insensitive or independent because no oxygen radicals are formed in the process. Single electron transfers are also biologically catalyzed, the reactions are termed oxygen-sensitive because the reactions lead to intermediates that will react with oxygen to form a superoxide anion and reform the parent compound (5). The futile cycle catalyzed by enzymes in the oxygen-sensitive series must be avoided for any potential sustainable pathway of TNT degradation.

A number of enzymes will catalyze two-electron transfer reductions on TNT (4, 15, 17, 25). NAD(P)H-dependent nitroreductases have received much attention due to their potential roles (including detrimental effects) in environmental degradation of nitroaromatic compounds (8, 11, 23, 27), and bacterial resistance to nitro-substituted antimicrobial compounds (20). Reports on aerobic bacterial TNT degradation have repeatedly demonstrated transformation of TNT to ADNTs, as well as the non-enzymatic condensation of reduction intermediates to dibenzylic azo- and azoxy-compounds by laboratory and environmental isolates. The specific enzymes catalyzing the reactions are less well documented, and anomalies in mass-balance and complications in identification of other transformation products has limited confidence in predicting or applying the process in nature.

The reduction of a nitro substituent to an amino group is considered a gratuitous reaction; no biodegradation pathways have been described that use the mechanism productively (9). Numerous reports have been published on the reduction of TNT to ADNTs and DANTs in bacterial cultures (10, 14, 19, 21, 26, 28). The amino-substituted compounds are generally noted as end products of the biological reactions, largely due to their stability relative to the partially reduced intermediate compounds. Since the reactions do not benefit the organisms and the intermediates are unstable, controlling or applying the reduction of TNT to a treatment process may be a difficult proposition.

The success of the proposed engineered pathway for sustained TNT degradation relies upon effective reduction to ADNT; otherwise significant loss of carbon to the organisms carrying out the process will result. The ADNT isomers will then serve as substrates for nitroarene dioxygenases (13). The identification of a nitroreductase that efficiently converts TNT to ADNT could prove useful in the construction of a TNT-degradation pathway in the event that our chosen host strain proved to be inefficient in this conversion. Several studies have shown that *E. coli* can reduce TNT to various products, including 4-amino-2,6-DNT (19, 29, 30), and one recent publication suggested that the oxygen-insensitive nitroreductases NfsA and NfsB might be the *E. coli* enzymes catalyzing the reaction (30). However, purified proteins or specific strains lacking *nfsA* and *nfsB* were not tested for their ability to reduce TNT, so the role of these proteins remains to be determined. Therefore, we decided to test whether the nitroreductases NfsA or NfsB were capable of reducing TNT to ADNT. We obtained and tested *nfsA*$^-$ and *nfsA*$^-$*nfsB*$^-$ *E. coli* strains for the ability to reduce TNT. We also tested the TNT-reducing ability of two *Pseudomonas* strains (*P. putida* KT2440, and *Pseudomonas* sp. strain Idaho) that might serve as good hosts for the constructed TNT degradation pathway. *P. putida* KT2440 is a certified safety strain that has been designated as a host for recombinant DNA applications (1). *Pseudomonas* sp. strain Idaho is a solvent-tolerant strain that might have a survival advantage in the presence of toxic nitroarene solvents (6). We cloned and over-expressed *nfsA* and *nfsB* in *P. putida* KT2440 and *Pseudomonas* sp. strain Idaho, and carried out the same TNT reduction studies in order to determine whether the addition of these genes would improve the TNT reducing ability of the potential host strains.

Methods

Cloning of *E. coli* nitroreductase genes. *E. coli* B strain ER2566 was used as the source of nitroreductase genes. Chromosomal DNA was prepared using a Puregene DNA isolation kit

(Gentra Systems, Minneapolis, Minn.). PCR was performed with the Expand Long Template PCR system (Roche, Indianapolis, Ind.). Oligonucleotide primers were designed based on sequences of *nfsA* and *nfsB* from *E. coli* K-12 derivatives available in the Genbank database (D38308; D25414). Primers used for amplification of *nfsA* were nfsAfor (5'-CCACCGCAATATTCACGTTCAG-3') and nfsArev (5'-GCGTATCATACATCGACGTGGC-3'); those for *nfsB* were nfsBfor (5'-CTTTCACATGGAGTCTTTATG-3') and nfsBrev (5'-GCAAGAGAGAATTACACTTCG-3'). The amplification was carried out by an initial denaturation at 95°C for 3 min followed by 25 cycles of 95°C for 1 min, 65°C for 1 min, 70°C for 1 min and then a final extension at 70°C for 5 min. PCR products of the appropriate sizes (0.7 kb) were obtained, purified from gels using a Qiaquick PCR purification kit (Qiagen, Chatsworth, CA), and ligated into *Sma*I-digested pK18. Plasmids were used to transform competent *E. coli* DH5□. Plasmids were isolated and sequenced to confirm the presence of *nfsA* and *nfsB*. The deduced amino acid sequence of NfsA was 100% identical to the *E. coli* K12 protein. The deduced amino acid sequence of NfsB was 98.6% identical to that from *E. coli* K12. *nfsA* and *nfsB* were also subcloned into the *Pst*I-*Sac*I digested broad-host-range vector pBBR-MCS5 (16). pBBR1-MCS5 and its derivatives were introduced into *Pseudomonas putida* KT2440 (22) and *Pseudomonas* sp. Idaho (6) by mating with *E. coli* host strains carrying the cloned genes.

Whole cell biotransformations. *E. coli* AB1157 (wild-type of *nfs* genes; parent of NFR402 and NFR502), NFR402 (*nfsA*$^-$), and NFR502 (*nfsA*$^-$*nfsB*$^-$) (3) were obtained from the *E. coli* Genetic Stock Center at Yale University. Each strain was grown at 37°C either in rich medium (LB) or in minimal medium with glucose as the sole carbon source. *E. coli* DH5□(pK18) (24), DH5□(pK18-*nsfA*), and DH5□(pK18-*nsfB*) were grown in the same way, except that kanamycin was added to a final concentration of 100 □g/ml. Cultures carrying pK18-based plasmids were treated with 100 □M IPTG when they reached mid-exponential phase (OD_{600}~ 0.4) for 3 h at 30°C in order to induce the expression of the cloned nitroreductase genes. *P. putida* KT2440 and *Pseudomonas* sp. Idaho carrying pBBR1-MCS5 and its derivatives with *nfsA* and *nfsB* inserted were grown at 30°C in LB medium containing gentamicin at a final concentration of 15 □g/ml. Cells were harvested by centrifugation, resuspended in minimal medium with 20 mM glucose and 0.05% TNT, and incubated at 30°C for approximately 18 h. Culture supernatants were collected and extracted with ethyl acetate, dried under vacuum, and the residue was dissolved in acetonitrile. The compounds were derivatized with bis(trimethysilyl)-trifluoroacetamide prior to analysis by GC-MS.

Results

The reduced TNT derivative 4-amino-2,6-DNT (4ADNT) was produced as the major product in biotransformation reactions with wild-type *E. coli* (Figure 1) as well as with the *nsfA*$^-$ and *nfsA*$^-$*nsfB*$^-$ mutants. No 2-amino-4,6-DNT was detected with any of the strains. Variable amounts of hydroxylamino-DNTs (HADNT) were generated in different experiments. In all of the reactions, a diaminonitrotoluene (DANT) product was also formed, most likely resulting from the reduction of 4ADNT. The only notable difference in product formation by the wild-type and the *nfsA*$^-$*nsfB*$^-$ mutant was a reduced level of DANT formation by the mutant strain. These results indicate either that 1) NfsA and NfsB do not participate in the initial TNT reduction, or 2)

that they do participate, but additional nitroreductases capable of catalyzing reduction of nitro groups are present in *E. coli*. In any case, the *E. coli* nitroreductases seemed to preferentially reduce the nitro group at the 4-position, which is consistent with the results of a recent report (30). We also cloned and expressed *nfsA* and *nfsB* in *E. coli* DH5 in order to determine whether we could improve TNT reduction activity by increasing the copy number and expression efficiency of the nitroreductase genes. Again, we obtained predominantly 4ADNT from TNT with or without cloned *nfsA* or *nfsB* present. However, the presence of multiple copies of *nfsA* and *nfsB* seemed to result in increased formation of HADNT.

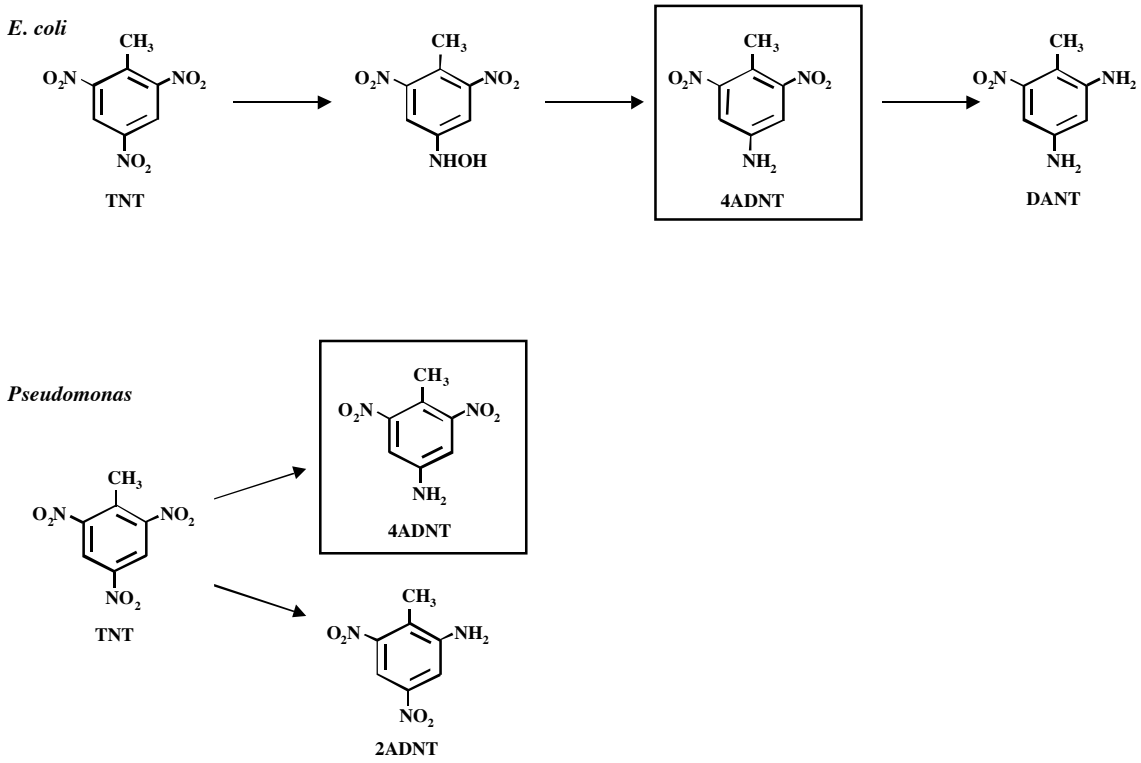


Figure VI-1. TNT reduction by *E. coli* and *Pseudomonas* strains. Major products are boxed.

The two *Pseudomonas* strains, *P. putida* KT2440 and *Pseudomonas* sp. Idaho were also capable of reducing TNT to ADNTs (Figure 1). Both strains preferentially produced 4ADNT, but they also were capable of making a small amount of 2-amino-4,6-DNT (~80:20 ratio). HADNTs and DANTs were not found, indicating that the reduction of the first nitro group went to completion and reduction of a second nitro group did not occur at a significant rate. Expression of *nfsA* or *nfsB* in either strain did not have a major effect on TNT reduction, except that small amounts of HADNTs or DANTs were sometimes detected as biotransformation products. The complete genome sequence of *P. putida* KT2440 has been determined (22), and it contains several possible nitroreductase genes, but it is not known at this time which nitroreductases contribute to TNT reduction.

Conclusions

It is not clear whether NfsA and NfsB can reduce TNT since all of the strains were able to reduce TNT to ADNTs either in the presence or absence of *nfsA* and *nfsB*. A definitive answer to this question will require the use of purified enzymes. Since introduction of *nfsA* or *nfsB* did not improve ADNT production in either an *E. coli* or *Pseudomonas* background, it appears that these genes will not be useful in our engineered TNT degrading strain. However, since all tested strains converted TNT to ADNTs, it is likely that the host chosen for the constructed pathway may have sufficient nitroreductase activity. We purposely used aerobic conditions for these experiments because the constructed TNT pathway will have at least two steps (initial ring dioxygenation of ADNT and aromatic ring cleavage) that require molecular oxygen as a substrate. TNT reduction would be expected to proceed further under anaerobic conditions, resulting in production of primarily DANTs and triaminotoluene (TAT). Based on the results obtained, *P. putida* KT2440 or *Pseudomonas* sp. Idaho may represent good host strains for an engineered TNT degradation pathway through 4ADNT. Both are capable of converting TNT to ADNTs, neither represents a known biosafety risk (*P. putida* KT2440 is a certified 'safe' strain (1)), and *Pseudomonas* sp. Idaho has the added advantage of solvent resistance. For a pathway that proceeds through 2ADNT, an alternative host may be needed. *Pseudomonas pseudoalcaligenes* JS45 is known to convert TNT to 2ADNT (12) and thus may be a good candidate.

Literature Cited

1. **Bagdasarian, M., R. Lurz, B. Ruckert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose cloing vectors II. Broad-host-range high copy number, RSF1010 derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene **16:**237-247.
2. **Bryant, C., and M. DeLuca.** 1991. Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. J. Biol. Chem. **266:**4119-4125.
3. **Bryant, D. W., D. R. McCalla, M. Leeksma, and P. Laneuville.** 1981. Type I nitroreductases of *Escherichia coli*. Can. J. Microbiol. **27:**81-86.
4. **Caballero, A., J. J. Lazaro, J. L. Ramos, and A. Esteve-Nunez.** 2005. PnrA, a new nitroreductase-family enzyme in the TNT-degrading strain *Pseudomonas putida* JLR11. Environ. Microbiol.**:**1211-1219.
5. **Cerniglia, C. E., and C. C. Somerville.** 1995. Reductive metabolism of nitroaromatic and nitropolycyclic aromatic hydrocarbons, p. 99-115. *In* J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Publishing Corp., New York.
6. **Cruden, D. L., J. H. Wolfram, R. D. Rogers, and D. T. Gibson.** 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. **58:**2723-2729.
7. **Ederer, M. M., T. A. Lewis, and R. L. Crawford.** 1997. 2,4,6-Trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J. Ind. Microbiol. Biotechnol. **18:**82-98.

8. **Esteve-Nuñez, A., A. Caballero, and J. L. Ramos.** 2001. Biological degradation of 2,4,6-trinitrotoluene. Microbiol. Mol. Biol. Rev. **65:**335-352.
9. **Esteve-Nuñez, A., A. Caballero, and J. L. Ramos.** 2001. Biological degradation of 2,4,6-trinitrotoluene, p. 335-352, Microbiology and Molecular Biology Reviews, vol. 65.
10. **Gilcrease, P. C., and V. G. Murphy.** 1995. Bioconversion of 2,4-diamino-6-nitrotoluene to a novel metabolite under anoxic and aerobic conditions. Appl. Environ. Microbiol. **61:**4209-4214.
11. **Haïdour, A., and J. L. Ramos.** 1996. Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. Environ. Sci. Technol. **30:**2365-2370.
12. **Hughes, J. B.** 2005. Novel Pathways of Nitroaromatic Metabolism: Hydroxylamine Formation, Reactivity and Potential for Ring Fission for Destruction of TNT - CU1214 Georgia Institute of Technology.
13. **Johnson, G. R., B. F. Smets, and J. C. Spain.** 2001. Oxidative transformation of aminodinitrotoluene isomers by multicomponent dioxygenases. Appl. Environ. Microbiol. **67:**5460-5466.
14. **Kalafut, T., M. E. Wales, V. K. Rastogi, R. P. Naumova, S. K. Zaripova, and J. R. Wild.** 1998. Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. Curr. Microbiol. **36:**45-54.
15. **Kitts, C. L., C. E. Green, R. A. Otley, M. A. Alvarez, and P. J. Unkefer.** 2000. Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6,-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). Can. J. Microbiol. **46:**278-282.
16. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-176.
17. **Kutty, R., and G. N. Bennett.** 2005. Biochemical characterization of trinitrotoluene transforming oxygen-insensitive nitroreductases from *Clostridium acetobutylicum* ATCC 824. Arch. Microbiol. **In press**.
18. **Kwak, Y. H., D. S. Lee, and H. B. Kim.** 2003. *Vibrio harveyi* nitroreductase is also a chromate reductase. Appl. Environ. Microbiol. **69:**4390-4395.
19. **McCormick, N. G., F. E. Feeherry, and H. S. Levinson.** 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Microbiol. **31:**949-958.
20. **Mendz, G. L., and F. Megraud.** 2002. Is the molecular basis of metronidazole resistance in microaerophilic organisms understood? Trends Microbiol. **10:**370-375.
21. **Naumova, R. P., N. N. Amerkhánova, and L. N. Zolotukhina.** 1984. Peculiarities of nitroreduction as a key stage in the microbial destruction of aromatic nitrocompounds. Appl. Biochem. Microbiol. **19:**400-404.
22. **Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodsen, H. Hilbert, V. A. P. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Düsterhöft, B. Tümmler, and C. Fraser.** 2002. Complete genome sequence and

comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**799-808.

23. **Pak, J. W., K. L. Knoke, D. R. Noguera, B. G. Fox, and G. H. Chambliss.** 2000. Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluoresce*ns I-C. Appl. Environ. Microbiol. **66:**4742-4750.
24. **Pridmore, R. D.** 1987. New and versatile cloning vectors with kanamycin-resistance marker. Gene **56:**309-312.
25. **Riefler, R. G., and B. F. Smets.** 2000. Enzymatic reduction of 2,4,6-trinitrotoluene and related nitroarenes: kinetics linked to one-electron redox potentials. Environ. Sci. Technol. **34:**3900-3906.
26. **Schackmann, A., and R. Müller.** 1991. Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. Appl. Microbiol. Biotechnol. **34:**809-813.
27. **Snellinx, Z., A. Nepovim, S. Taghavi, J. Vangronsveld, T. Vanek, and D. van der Lelie.** 2002. Biological remediation of explosives and related nitroaromatic compounds. Environ. Sci. Pollut. Res. Int. **9:**48-61.
28. **Vasilyeva, G. K., B.-T. Oh, S. P.J., R. A. Drijber, V. D. Kreslavski, R. Minard, and J.-M. Bollag.** 2000. Aerobic TNT reduction via 2-hydroxylamino-4,6-dinitrotoluene by *Pseudomonas aeruginosa* strain MX isolated from munitions-contaminated soil. Biorem. J. **4:**111-124.
29. **Yin, H., T. K. Wood, and B. F. Smets.** 2005. Reductive transformation of TNT by *Escherichia coli* resting cells: kinetic analysis. Appl. Microbiol. Biotechnol. **In press**.
30. **Yin, H., T. K. Wood, and B. F. Smets.** 2005. Reductive transformation of TNT by *Escherichia coli*: pathway description. Appl. Microbiol. Biotechnol. **67:**397-404.

VII. Metabolism of TNT by *Burkholderia cepacia* JS872

Background

Previous work (unpublished results) with the 2,4-DNT-degrading strain *Burkholderia* sp. strain DNT showed that TNT was cometabolized by cultures that were actively growing on 2,4-DNT. Cultures presented with 200 µM 2,4-DNT and 10 µM TNT, transformed TNT only as long as 2,4-DNT remained in the culture medium. The transformation products were not identified, but the results suggested that other DNT-degrading strains might transform TNT during growth on DNT. *Burkholderia cepacia* JS872 exhibits the most rapid growth on 2,4-DNT of any strain isolated to date (2). Strain JS872 was tested for the ability to cometabolize TNT during growth on 2,4-DNT.

Results and Discussion

A culture of *B. cepacia* JS872 was grown in minimal medium (BLK) (1) with 2,4-DNT provided as the sole carbon and nitrogen source. The cells were harvested by centrifugation, washed with BLK, and suspended in BLK to an OD_{600} ≈1. Mixtures of 200 µM 2,4-DNT with 20 µM TNT, 2ADNT, or 4ADNT were incubated with the cells for 1 h at 30 °C with shaking at 200 rpm. Analysis by HPLC revealed that 2,4-DNT was degraded by all cultures, 4ADNT was not transformed, and that TNT and 2ADNT were transformed to novel nonpolar yellow metabolites with identical retention times and UV-Vis spectra. Abiotic controls did not transform any compound.

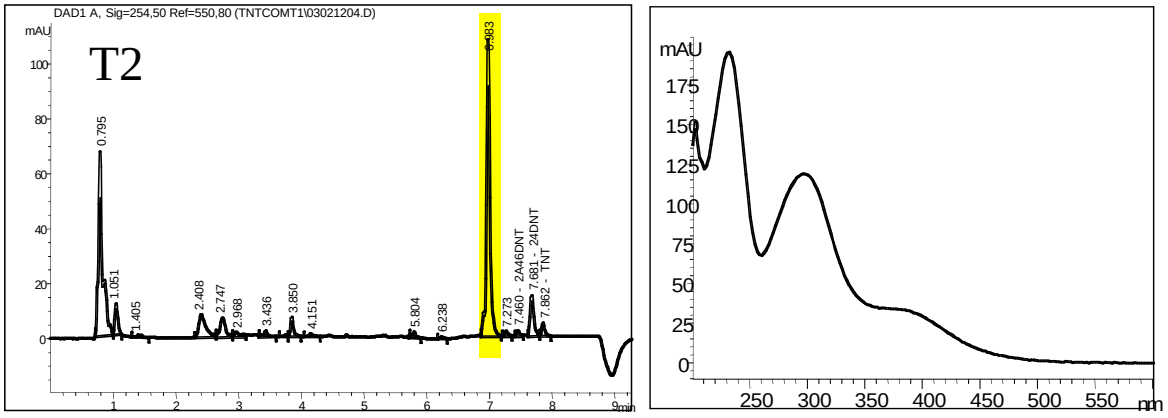


Figure VII-1. Novel yellow metabolite (marked in yellow) detected after incubation of *B. cepacia* JS872 with mixture of 2,4-DNT and either TNT or 2ADNT. UV-Vis spectrum (right panel) and retention times were identical for products derived from TNT and 2ADNT.

The transformation of 2ADNT and TNT to the same compound suggests that TNT is reduced to 2ADNT prior to conversion to the yellow metabolite. It then follows that *B. cepacia* JS872 and possibly other 2,4-DNT-degrading bacteria contain nonspecific nitroreductase(s) that catalyze the reduction of the nitro group at the 2-carbon position. In a related SERDP project (CU-1214 Novel pathways of nitroaromatic metabolism: hydroxylamine formation, reactivity and potential for ring fission for destruction of TNT) we found that the nitrobenzene-degrading strain *Pseudomonas pseudoalcaligenes* JS45 constitutively expresses a nonspecific nitroreductase that attacks TNT at the 2-carbon position which leads to the formation a very polar yellow metabolite distinct from the yellow metabolite described from strain JS872. *P. aeruginosa* strain MX(3) also preferentially reduced TNT at the *ortho* position, but no further transformation of 2ADNT other than azoxy formation was reported. Such transformations found in multiple microorganisms suggests that nitroreductases that are specific for the 2-carbon position may be more common than previously thought (3).

The results with *B. cepacia* JS872 further suggest that genetic engineering to insert a nitroreductase into 2,4-DNT-degrading strains to initiate the reduction of TNT to an aminodinitrotoluene may not be necessary. Further work is required to at a minimum, survey other DNT-degrading strains for TNT-reduction activity, establish the rate of the nitroreduction reaction, and determine whether the activity is constitutive.

Literature Cited

1. **Bruhn, C., H. Lenke, and H.-J. Knackmuss.** 1987. Nitrosubstituted aromatic compounds as nitrogen source for bacteria. Appl. Environ. Microbiol. **53:**208-210.
2. **Nishino, S. F., G. Paoli, and J. C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66:**2139-2147.
3. **Vasilyeva, G. K., B.-T. Oh, P. J. Shea, R. A. Drijber, V. D. Kreslavski, R. Minard, and J.-M. Bollag.** 2000. Aerobic TNT reduction via 2-hydroxylamino-4,6-dinitrotoluene by *Pseudomonas aeruginosa* strain MX isolated from munitions-contaminated soil. Bioremed. J. **4:**111-124.

VIII. Aerobic transformation of aminomethylnitrocatechol isomers – Towards an engineered pathway for aerobic TNT degradation

Background

There is no naturally occurring pathway for complete aerobic degradation of 2,4,6-trinitrotoluene (TNT) (13), but TNT and its metabolites might be transformed by co-metabolic reactions that are not coordinated in a single microbe. A challenge is to link the catabolic enzymes to provide a sustainable pathway for TNT degradation. Our first approaches to the problem evaluated whether oxygenases from nitroarene degradation pathways exhibit the catalytic promiscuity to accommodate and transform metabolites from TNT. The rationale is summarized in Figure VIII-1. The figure displays a series of potential dioxygenase and monooxygenase catalyzed transformations of aromatic substrates similar to 2,4-DNT and 2,6-DNT degradation routes (3, 5, 10, 16). A key to the strategy is the gratuitous reduction of TNT to ADNT isomers. The partial reduction of TNT relieves the collective effects of the three electron-withdrawing nitro groups, allowing hydroxylation of ADNTs by multicomponent nitroarene-dioxygenases (7). Each subsequent removal of a nitro group or transformation through oxidative attack could open the molecule to further degradation, or that transformation could form a dead-end metabolite. This stage of the project explored the two options.

Synthesis and characterization of 3-amino-4-methyl-5-nitrocatechol and 3-amino-6-methyl-5-nitrocatechol. The specific transformation of 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT) to yield aminomethylnitrocatechols and concomitant removal of a nitro substituent from the ring revealed a potential route for bacteria to degrade TNT under aerobic conditions (7). The next logical question is whether those catechols are susceptible to further biological degradation. Chemical synthesis of the compounds is prohibitively complex and expensive to provide substrates for experiments (R. Spanggord, Personal Communication). Methods were developed for biologically producing the aminomethylnitrocatechols and purifying the products from larger-scale reactions. The recovered, pure product was used for subsequent experiments to test biological degradation of the catechols. The product from 24DNTDO transformation of 2ADNT was analyzed using proton and ^{13}C NMR to confirm structure of the compound.

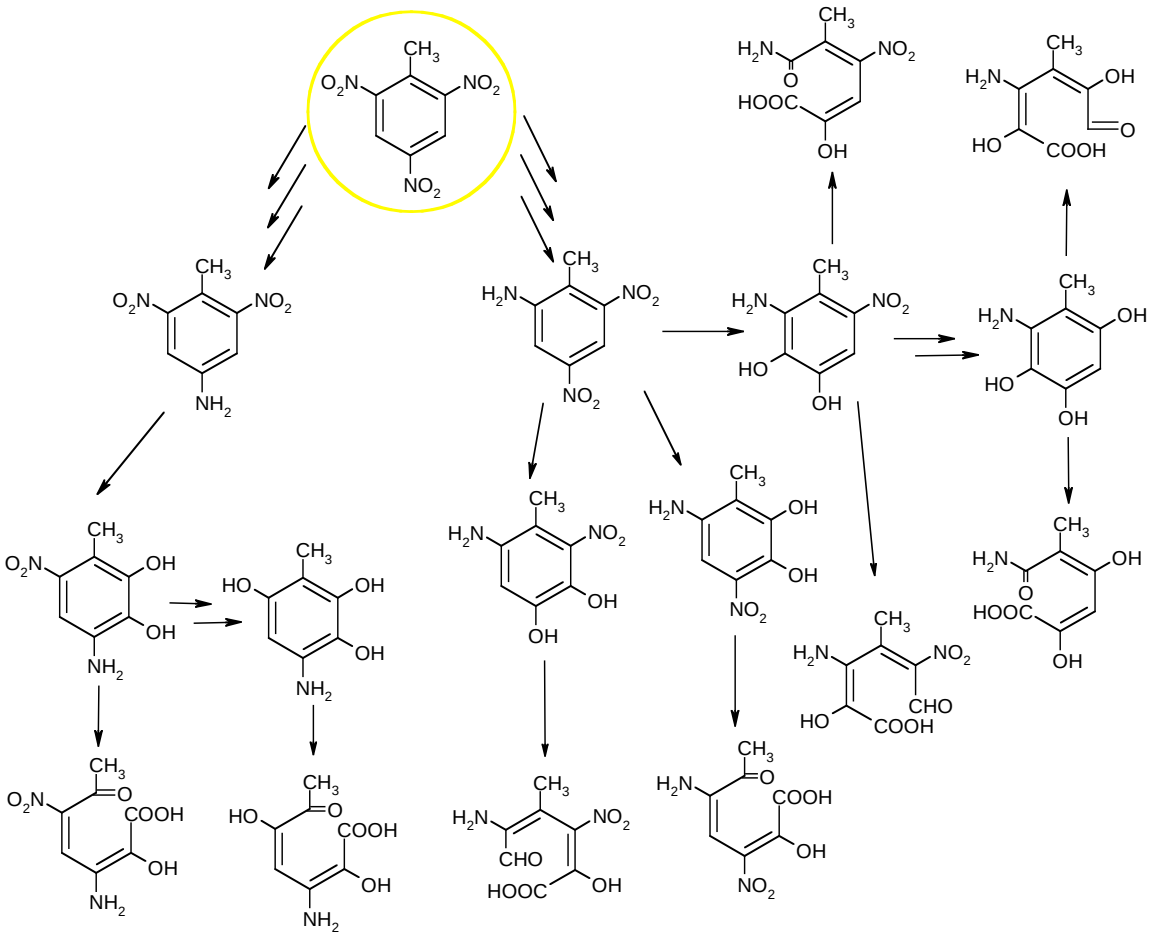


Figure VIII-1. Potential reduction and oxygenase catalyzed transformation of 2,4,6-trinitrotoluene (circled) and metabolites.

Methods

Subcloning the 2,4-DNT dioxygenase and nitrobenzene dioxygenase operons. The dioxygenase operons encoding the nitrobenzene dioxygenase from strain JS765 and the 2,4-DNT dioxygenase from strain DNT were subcloned into plasmid vectors pK19 and pK18, respectively. The pK plasmids encode for kanamycin resistance and allow inducible expression of recombinant genes from a *lac* operator in the multiple cloning region (12). The 2,4-DNT dioxygenase operon was excised from pJS48 (17) as a 6.2-kb *Sst*I:*Sal*I restriction fragment. The nitrobenzene dioxygenase operon was obtained from pDTG927 (9) as a 4.7-kb *Sst*I:*Eco*RI restriction fragment. The oxygenase encoding fragments were ligated with compatibly cleaved plasmid DNA then transferred to *E. coli* DH5. After screening and confirmation of structures, the plasmid containing the 2,4-DNT dioxygenase was designated pJS1048, and the plasmid containing the nitrobenzene dioxygenase genes was designated pJS1927.

Whole cell transformations. The recombinant strains were cultivated in LB media containing glycerol (1.0%) and kanamycin (20 mg/L). The initial cultures (25 ml in 20 ml baffled flasks)

were inoculated with a single colony and incubated 8-10 h at 37˚ with shaking. The 25 ml culture was transferred to 375 ml media in a 1.8 L Fernbach flask and incubated 12-14 h at 37˚ with shaking. The overnight culture was transferred to 12 L of the LB-glycerol medium that was buffered with 10 mM potassium phosphate (pH 6.8) in a New Brunswick microferm fermenter (New Brunswick Scientific, Edison NJ). The culture was incubated with constant aeration (15 L/min) and agitation (350 rpm) and growth was monitored by measuring cell density. When the A_{600} reached 0.8, IPTG (final conc. 0.5 mM) was added to induce synthesis of the oxygenases, then incubated 8-12 h. The cells from the fermenter were harvested using centrifugation and washed with phosphate buffer. Following the wash, the cells were suspended in phosphate buffer, then the A_{600} of the suspension was determined.

In the transformation reactions, the cells were transferred to 5-10 L of phosphate buffer (20 mM, pH 6.5) to yield an apparent A_{600} of 4-5. The ADNT substrate (200 □M) and pyruvate (1 mM) were added to the cell suspension to initiate the transformation reaction and provide energy source for NADH regeneration. Samples were taken at regular intervals to monitor the progress of the reaction. When the substrate was entirely transformed or oxygenase activity had ceased, ascorbate (5 mM) was added to limit decomposition of the catechol, the cells were removed by centrifugation and the supernatant saved for isolation of the aminomethylnitrocatechol. The supernatant was stored at 4º for short terms (< 48 h) or -70º for longer term storage.

Isolation of aminomethylnitrocatechol. A number of solid phase extraction methods were tested for use in isolation of AMNCs from the bulk reaction supernatant. A series of commercial pre-packed columns included, Sep-Pak C-18, Sep-Pak C -8, and Porapak-RDX (Waters Corp., Milford, Mass). A mobile phase of acetonitrile:water was used for elution of substrate and products from the commercial cartridges. The eluate was analyzed using HPLC and fractions containing aminomethylnitrocatechols were extracted with ethyl acetate to concentrate product. The second series of solid phase matrices were made using various forms of Brockman-type aluminum oxide (Aldrich Chem., Milwaukee, Wisc.). The alumina was equilibrated prior to use by repeated washings with potassium phosphate buffer (20 mM, pH 7.0). Once equilibrated, aminomethylnitrocatechol binding was tested in batch and column binding experiments. For batch experiments, the catechol-containing solution was stirred with a 10% (wt/vol) suspension of alumina. For column testing, 1.5 cm beds of washed alumina were poured in 1□8 cm plastic cartridges. Larger columns and beds were used for purification of the product using the parameters determined using the small cartridges. Weakly binding compounds were chromatographed using mobile phase of water:acetonitrile. Bound catechol was eluted from the alumina with acidified acetone and the solvent removed under vacuum. When a pure aminomethylnitrocatechol isomer was obtained (purity tested by HPLC) the crystals were dried in a vacuum desiccator to remove any remaining solvent. Product was stored under nitrogen and desiccated at -20º C.

Analysis. Oxygenase activity was determined by measuring the nitrite in the reaction supernatant, catechol accumulation, and ADNT transformation. Nitrite concentrations were measured using standard methods. Catechol and ADNT concentrations were monitored using reverse phase HPLC (Supelcosil, ABZ+plus column (Supelco, Belefonte, PA) with a mobile phase of acetonitrile and 0.1% trifluoroacetic acid). Protein concentrations were determined

using the Pierce bichinchonic acid assay reagents (Rockford, IL). NMR analyses were done by the analytical core facility at the University of Iowa (Iowa City, IA).

Results and Discussion

Transformation of 4-amino,-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. The nitrobenzene dioxygenase and the 2,4-DNT dioxygenases from strain DNT catalyzed the specific dioxygenation of the aminodinitrotoluene isomers (7). Transferring the two oxygenase operons to the pK-vector series did not markedly increase oxygenase specific activity compared to other expression vectors. There was a benefit in the kanamycin selection over penicillin derivatives since it is not degraded during bacterial growth so the selection for recombinant bacteria is maintained. Further experiments that test other *E. coli* host strains, growth, or induction conditions might identify steps to increase oxygenase activity.

The transformation reaction with 2ADNT by *E. coli* DH5 (pJS1048) showed a rapid, nearly stoichiometric transformation of the substrate to the catechol (tentatively identified as 3-amino-4-methyl-5-nitrocatechol). The oxygenase specific activity was much lower from *E. coli* DH5 (pJS1927). To overcome the problem, multiple additions of catalyst (recombinant bacteria) were made to sustain the oxygenase reaction and produce adequate product levels for purification.

Isolation and purification of aminomethylnitrocatechols. The three polar substituents on the ring caused some difficulties for product isolation. Interactions with commercial reverse phase resins were quite weak and encouraged the trials using alumina matrices. The Brockman alumina derivatives were reported to be highly effective for collection of catecholic products in other biocatalytic processes (14). With the aminomethylnitrocatechols, only acidic alumina differentially bound the aminomethylnitrocatechol and ADNT. Neutral, weakly acidic, and basic preparations of the alumina all failed to bind the catecholic products. The acidic preparation bound the product exceptionally well; of the solvents tested, a 1:1 solution of acetone and HCl (0.1 N) provided best elution of the product. The untransformed aminodinitrotoluene was removed readily from the matrix with a weaker solvent, leaving the catechol, which was subsequently collected.

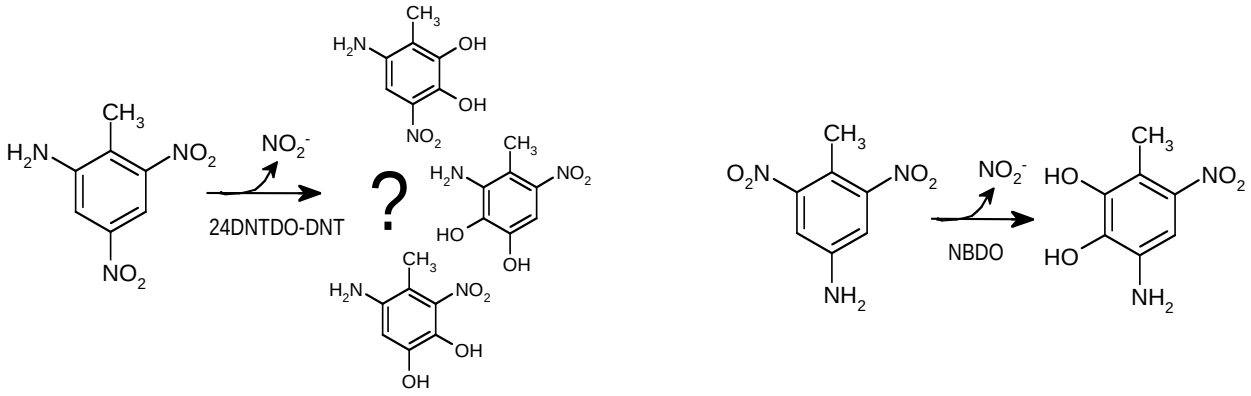
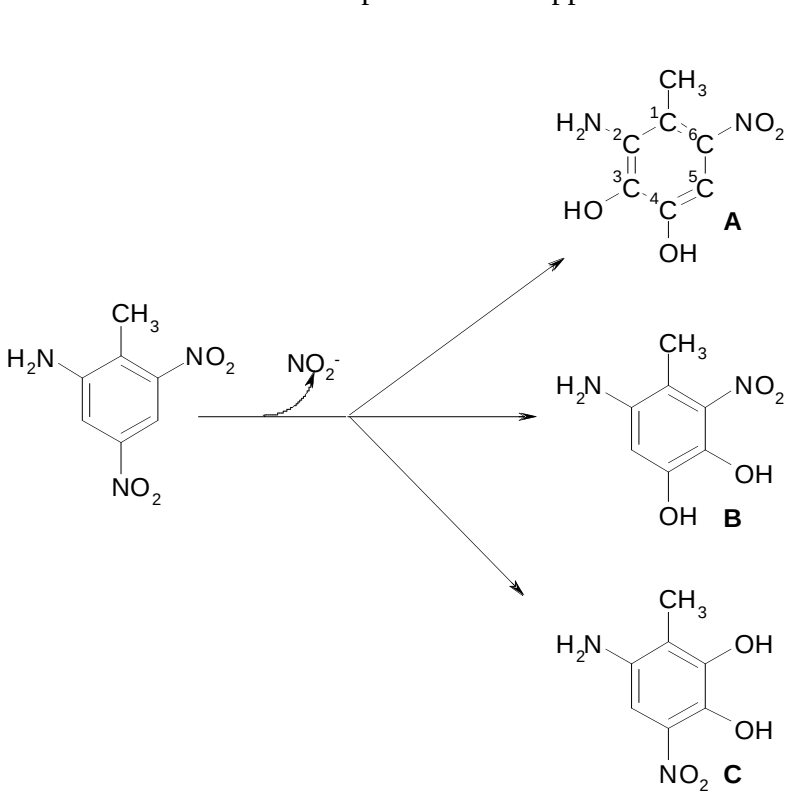


Fig VIII-2. Potential dioxygenase attacks of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene.

NMR analysis of product from 2-amino-4,6-dinitrotoluene. It is important that the products from transformation of the ADNTs are unequivocally identified so the appropriate enzymes are selected for subsequent reactions. The reaction with nitrobenzene dioxygenase and the symmetrical 4ADNT could only yield one catechol isomer following oxygenase attack and nitrite removal (Figure VIII-2). There was some uncertainty to the structure of the aminomethylnitrocatechol in the reaction with the 2,4-DNT dioxygenase and 2-ADNT. Three aminomethylnitrocatechol isomers could result from dioxygenase-catalyzed transformation and denitration of 2ADNT (Figure VIII -2).

The product from dioxygenase-catalyzed transformation of 2ADNT was analyzed by 13C and proton NMR to confirm its structure (Figure VIII-3). The proton shift data do not support structure B, as the benzylic proton shift of 6.95 ppm was much higher than that predicted for the structure. The measured ^{13}C shifts correlate well with those predicted for structure A, less so with structure C. Additionally other work with 2,4-DNT dioxygenase indicates that the enzyme does not attack a nitro substituent adjacent to the methyl group in reactions with other nitroarenes. The results and interpretation all support structure A as the reaction product.



Predicted Chemical Shifts

	^{13}C	^{1}H
A	1-113.4	
	2-136.9	
	3-137.1	
	4-143.2	
	5-102.1	6.73
	6-143.9	
	CH_3-13.3	2.35
B	1-113.4	
	2-142.3	
	3-109.7	6.03
	4-143.2	
	5-129.5	
	6-138.5	
	CH_3-13.3	2.35
C	1-119.0	
	2-142.3	
	3-104.1	6.77
	4-134.8	
	5-129.5	
	6-146.9	
	CH_3-13.3	2.35

Observed Chemical Shifts

^{13}C - 110.9, 136.8, 137.1, 142.9, 108.7, 143.5, 14.2

^{1}H - 6.95, 2.26

Figure VIII-3. Potential dioxygenase catalyzed transformation of 2ADNT by 2,4-DNT dioxygenase and predicted chemical shifts and results of NMR analysis of the purified product.

Transformation of 3-amino-4-methyl-5-nitrocatechol by the MNC-monooxygenase of 2,4-DNT pathway

Bacteria gratuitously reduce TNT under aerobic conditions to yield the stable reduction products, 2ADNT and 4ADNT. Our preceding work showed that nitroarene dioxygenases catalyze hydroxylation of the ADNT isomers to yield nitrite and aminomethylnitrocatechols (AMNC). The structures are similar for the product from 4ADNT (3-amino-4-methyl-5-nitrocatechol) and the catechol intermediate of the 2,4-DNT pathway (Figure VIII-4). The similarity might allow the amino-substituted compound to be a substrate for the enzyme from the 2,4-DNT pathway.

4-Methyl-5-nitrocatechol monooxygenase (MNCMO) catalyzes hydroxylation of 4-methyl-5-nitrocatechol in the 2,4-DNT pathway to yield 2-hydroxy-5-methyl-quinone and concomitant removal of the nitro group from the substrate (5). MNCMO is a single component, flavin-containing enzyme that loosely fits in the phenol hydroxylase enzyme family (8 5768). The substrate preference is fairly restricted, but not solely limited to the physiologically-relevant, 4-methyl-5-nitrocatechol (4, 11). MNCMO are available from two 2,4-DNT degrading bacterial strains (*Burkholderia cepacia* R34 (10) and *Burkholderia* sp. strain DNT (16)). The enzymes were tested for the transformation of 3-amino-4-methyl-5-nitrocatechol. Reaction rates were determined and products were characterized to evaluate the potential for the MNCMOs in a pathway for TNT degradation.

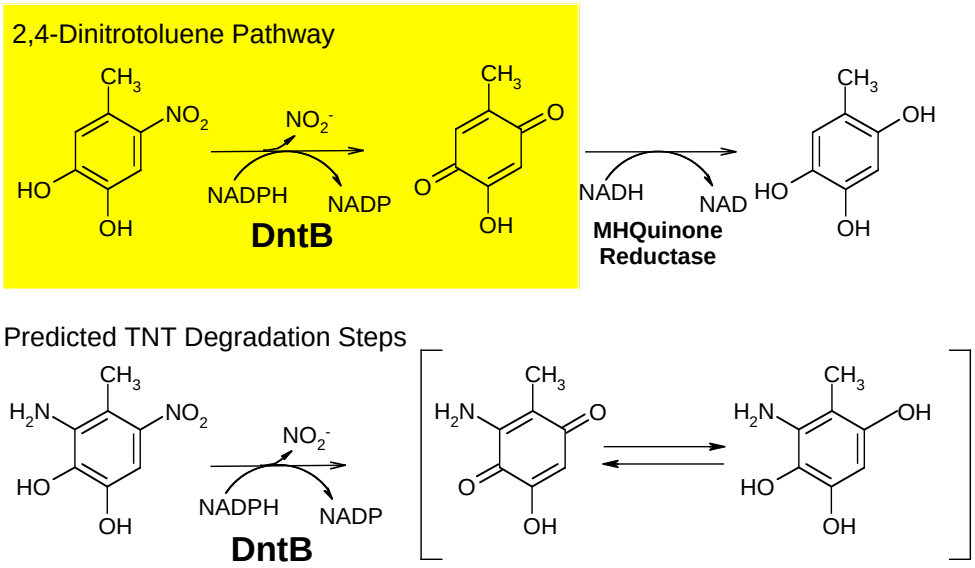


Figure VIII-4. Monooxygenase-catalyzed transformation of methylnitrocatechol and aminomethynitrocatechol.

Methods

MNC monooxygenase assays. *E. coli* JM109 (pJS338), a recombinant strain that carries the gene for the MNCMO from *Burkholderia cepacia* R34, and *E. coli* JM109 (pJS59) a recombinant strain that carries the gene for the MNCMO from *Burkholderia* sp. strain DNT were used as enzyme sources for assays. MNCMO activity was measured spectrophotometrically by monitoring the disappearance of MNC at 420 nm (_ = 5.97 mM^{-1} cm^{-1} at pH 7.5) (4) or the disappearance of 3A4M5NC at 463 nm. (_= 5.91 mM^{-1} cm^{-1} at pH 7.5). The assay cuvette contained NADPH (200 □M), MNC or 3A4M5NC (2-20 □M), and appropriate amounts of cell lysate in 1 ml of KH_2PO_4 buffer (0.05 M, pH 7.5). The reaction was started by the addition of MNC or AMNC. Specific activities are expressed as micromoles of substrate transformed per minute per milligram of protein. NADPH oxidation was monitored spectrophotometrically by the decrease in absorbance at 340 nM. Protein concentrations were determined using the Pierce bichinchonic acid assay reagents (Rockford, IL).

Qualitative assessment of MNCMO transformation of 3A4M5NC was done spectrophotometrically by monitoring the reaction spectral changes with time from 190-500 nm. Glucose-6-phosphate dehydrogenase and glucose-6-phosphate were included in qualitative assay mixtures to regenerate limiting concentrations of NADPH and to eliminate the interference to the spectrum due to NADPH oxidation.

In the assays monitoring whole-cell transformation of substrate, the catechol transformation was measured using reverse phase HPLC. Nitrite accumulation in the medium was determined using standard methods.

Ring Fission Assays. The ring-fission enzyme from the 2,4-DNT pathway, 2,4,5-trihydroxytoluene oxygenase (THTO), was provided in cell free extracts from *E. coli* (pJS333) (6). The substrate was enzymatically produced using the MNCMO from strain R34. Transformation of 2-amino-3,4,6-trihydroxytoluene by THTO was monitored spectrophotometrically, and the end products were analyzed neat by reverse phase HPLC and LC-MS. The products were also derivatized using dinitrophenylhydrazine (15) and analyzed using LC-MS.

Chromatography. Reverse phase HPLC was done using a Supelcosil, ABZ+plus column (Supelco, Belefonte, PA) with a mobile phase of acetonitrile and 0.1% trifluoroacetic acid. A gradient of mobile phase strength was used to optimize substrate and product resolution. Product analysis using LC-MS was done using ThermoFinnigan LCQ Advantage System. The analytes were separated using a Phenomenex Luna C18 column (Phenomenex Corp., Torrence, Calif). Mass spectrometer operating parameters for hydroxymethylquinone and amino-trihydroxytoluene analysis were established by tuning instrument using freshly sublimated 4-methylcatechol. The parameters for analysis of ring fission products were established using dinitrophenylhydrazine-derivatized propionaldehyde as the reference compound for tuning the instrument.

Results and Discussion

The initial experiments tested 3A4M5NC transformation by recombinant strains carrying the MNC monooxygenase gene. In the reactions, 3A4M5NC (HPLC elution time 9.8 min) was transformed rapidly to a more polar compound (HPLC elution time 8.2 min). Nitrite also accumulated as a product, indicating oxidative transformation of the substrate. The production of nitrite did not correlate perfectly with 3A4M5NC disappearance; the stoichiometry was only 0.5-0.7 for the reactions.

The assays comparing activity 3A4M5NC and 4M5NC as substrates for MNCMO showed that the relative activity was comparable for the two substrates (Table VIII-1). The result suggests the additional amino-substituent does not significantly affect catalysis, and is consistent with the Haigler et al. hypothesis that the hydroxyl groups meta- and para to the nitro group are the critical moieties for the MNCMO-substrates (4). The specific activity was significantly higher for the monooxygenase from strain R34 than that from strain DNT, the apparent difference in rate was likely worsened by substrate inhibition found with the MNCMO from strain DNT (11). Regardless, the differences indicate that any engineered pathway should make use of the monooxygenase from strain R34.

Table VIII-1. Substrate Preference –Methylnitrocatechol Monooxygense

Oxygenase Source	Activity	
	4M5NC	3A4M5NC
Strain DNT	2.75	3.53
Strain R34	72.2	68.2

A subsequent experiment and group of analyses tracked the transformation of 3A4M5NC and analyzed the reaction products. Analysis of the reaction spectra indicated direct conversion of substrate to product (Figure VIII-5). The pair of isosbestic points at 363 nm and 272 nm are consistent with absence of any intermediate in the reaction. Nitrite accumulated in the reaction supernatant consistent with oxidation reaction proposed in figure inset (Figure VIII-5) and analogous to the reaction in the 2,4-DNT pathway. Analysis of the reaction using LC-MS confirmed the formation of 2-amino-6-hydroxy-3-methylquinone (Figure VIII-6). A molecular ion of 152 amu was identified using negative APCI mode (Figure VIII-6 (left)) corresponding to the aminohydroxymethylquinone. Following chemical reduction of the product using sodium borohydride, the product was analyzed using MS-MS in the positive APCI mode. The molecular ion shifted to 156 amu as anticipated for the corresponding aminotrihydroxytoluene (Figure VIII-6 (right)). Principal ion fragments in the MS-MS result were also consistent with formation of the aromatic triol.

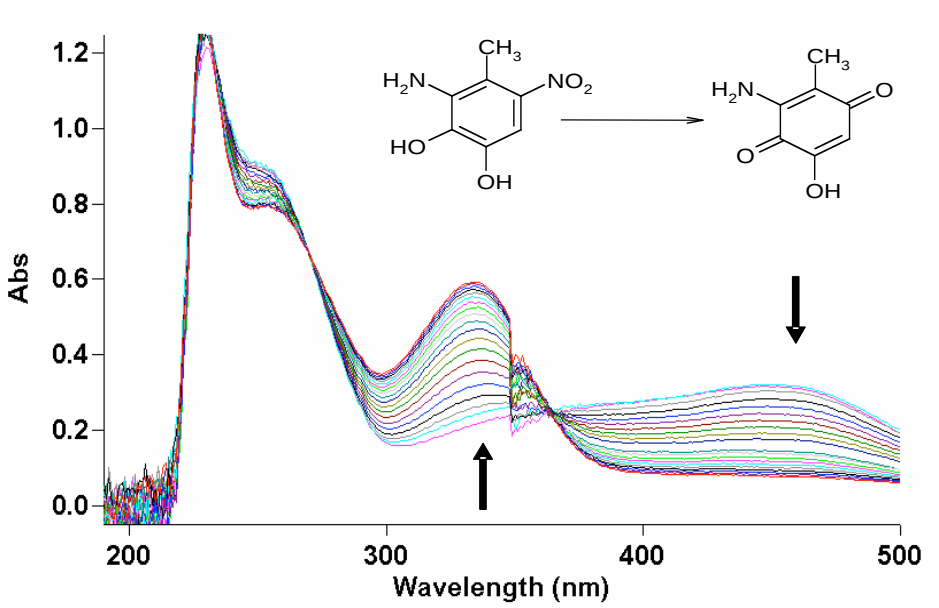


Figure VIII-5. Spectral changes associated with transformation of 3-amino-4-methyl-5-nitrocatechol by MNCMO. Spectra were recorded every 60 sec. Arrows indicate disappearance of 3A4M5NC and concomitant accumulation of product.

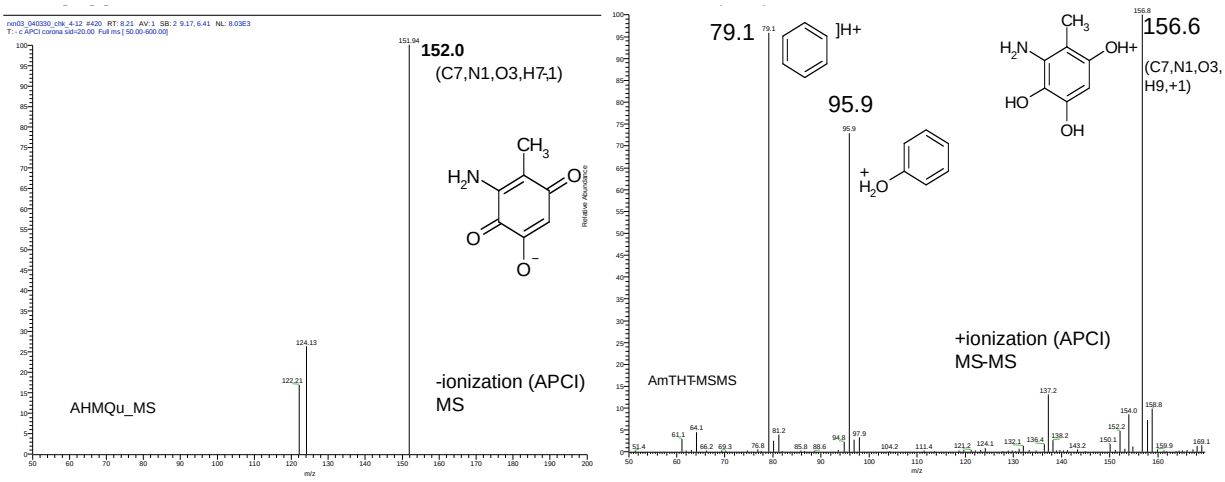


Figure VIII-6. LC-MS and LC-MSMS analysis of product from the MNCMO-3A4M5NC reaction.

The production of the aminotrihydroxytoluene brought us to another possible step in the oxidative pathway for TNT degradation. Again the amino-substituted analog was similar to the physiological substrate in the 2,4-DNT pathway, suggesting it might be to susceptible to transformation (Figure VIII-7). Incubation of the amino-triol caused a disappearance of the substrate absorbance at 335 nm and a gradual shift in the spectrum to yield a broad (260-330 nm), weakly absorbing peak (not shown). Analysis of the product by reverse phase-HPLC

revealed that the amino-THT was fully consumed during the reaction and a putative product peak was detectable (Figure VIII-8). No transformation of the amino-THT reaction was detected in control reactions with *E. coli* cell extracts that did not include the *dntD*-gene product. No further characterization of the product was obtained using LC-MS. It is unknown if the product did not ionize using the present conditions or if other spontaneous decomposition blocked analysis.

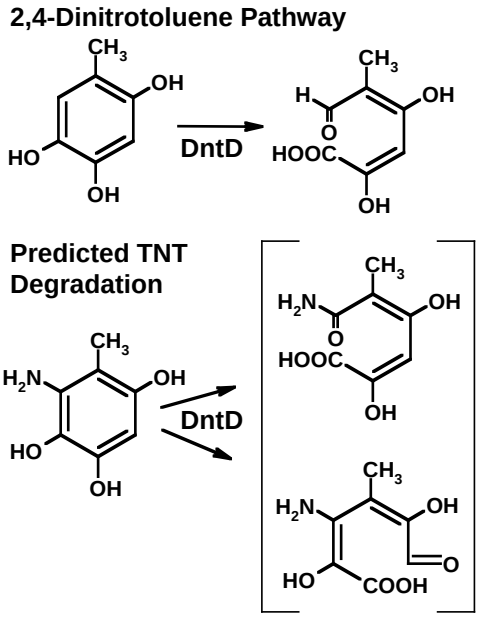


Figure VIII-7. Ring fission step in the 2,4-DNT pathway and possible transformation of amino-THT by THT-oxygenase.

Conclusions

Our work examining degradation of 3-amino-4-methyl-5-nitrocatechol traversed one branch of routes for aerobic TNT degradation (Figure VIII-9). We established clear evidence for oxidation of 3A4M5NC by methylnitrocatechol monooxygenases. The step removed the remaining nitro group from the ring yielding nitrite and 3-amino-5-hydroxy-2-methyl-[1,4]-benzoquinone. Chemical or enzymatic reduction of the substituted benzoquinone produced 2-amino-3,4,6-trihydroxytoluene. The reaction produced an intermediate without nitro groups and provided a substrate for potential ring fission and subsequent degradation of the molecule. Results of ring-fission experiments were equivocal. It is evident that the THT-oxygenase transformed 2-amino-3,4,6-trihydroxytoluene, but quantitation of the reaction and identification of the product(s) has not been attained. Clarifying the ring fission step is an important key to establishing the enzymatic fitness of a strain designed to carry out the process.

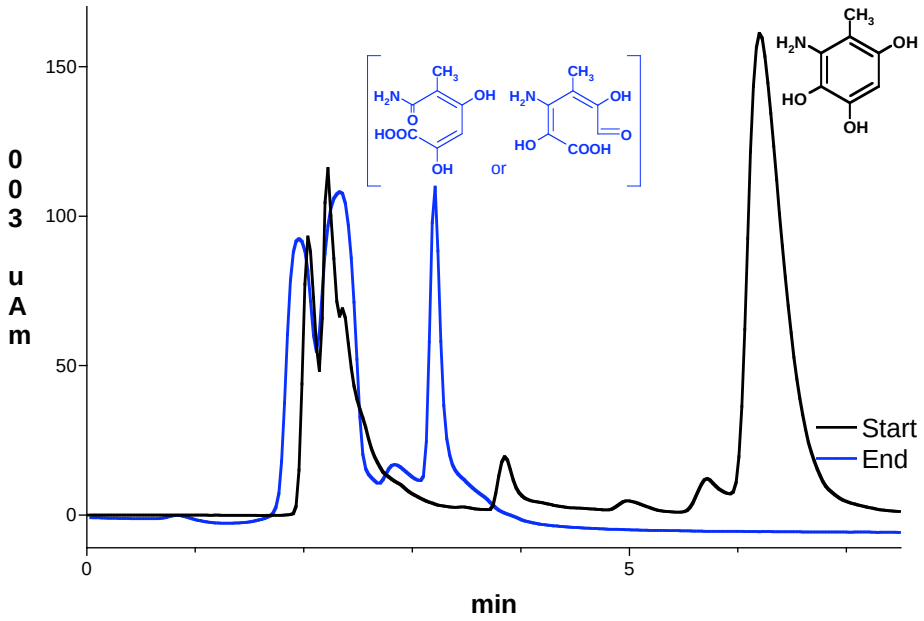


Figure VIII-8. Transformation of 2-amino-3,4,6-trihydroxytoluene by THTO. Black chromatogram shows substrate (ATHT) reaction mixture prior to incubation with THTO. Blue chromatogram shows analysis of the reaction following a 20 min incubaction with cell extract containing THTO.

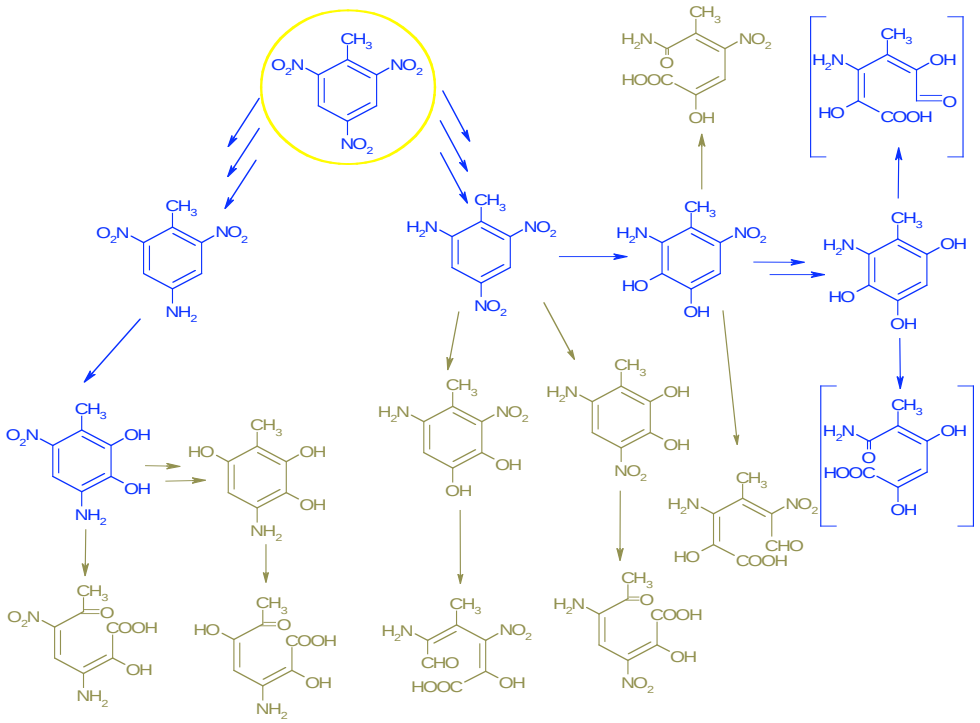


Figure VIII-9. Potential reduction and oxygenase catalyzed transformation of 2,4,6-trinitrotoluene (circled) and metabolites. Routes in blue indicate steps and products identified to date. The bracketed compounds are proposed based on enzyme activity with analogous substrates. Multiple arrows between compounds indicate intermediate compounds not shown.

Another critical consideration to harnessing the proposed degradative routes is the potential loss of substrates and intermediates via non-enzymatic reactions. During the reductive steps, the partially reduced nitroso-intermediate is particularly apt to react with nucleophiles in its environment (1). Next, the substituted anilines are also subject to nucleophilic addition and coupling reactions with other organic molecules. This type of reaction has been demonstrated with aniline and benzoquinone as model compounds in previous studies (2). The intermediates we have identified in our pathways will be subject to similar reactivity. In laboratory studies, that reactivity can complicate analysis of reaction products and determining reaction kinetics; furthermore, there is potential for enzyme inactivation through similar nucleophilic additions to the protein. In an environmental application of the process, the intermediates would be subject to extensive sorption to soils and sediments, potentially limiting the effectiveness of degradation.

The potential problems for the hybrid pathway might be overcome with genetic engineering of the system. An effectively engineered operon could relieve the bottleneck that allows accumulation of reactive intermediates. The relief might require coordinated expression of coupled steps or elevated levels of an enzyme that limits transformation. A component that is missing in our route is the hydroxy-benzoquinone reductase from the 2,4-DNT pathway. Identifying an enzyme and corresponding gene for that step could be critical to funneling the metabolites to ring fission and the lower pathway.

Literature Cited

1. **Ahmad, F., and J. B. Hughes.** 2002. Reactivity of partially reduced arylhydroxylamine and nitrosoarene metabolites of 2,4,6-trinitrotoluene (TNT) toward biomass and humic acids. Environ. Sci. Technol. **36:**4370-4381.
2. **Colon, D., E. J. Weber, and G. L. Baughman.** 2002. Sediment associated reactions of aromatic amines. 2. QSAR development. Environ. Sci. Technol. **36:**2443-2450.
3. **Haigler, B. E., G. R. Johnson, W.-C. Suen, and J. C. Spain.** 1999. Biochemical and genetic evidence for *meta*-ring cleavage of 2,4,5-trihydroxytoluene in *Burkholderia* sp. Strain DNT. J. Bacteriol **181:**965-972.
4. **Haigler, B. E., W.-C. Suen, and J. C. Spain.** 1996. Purification and sequence analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp. strain DNT. J. Bacteriol. **178:**6019-6024.
5. **Haigler, B. H., S. F. Nishino, and J. C. Spain.** 1994. Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. J. Bacteriol. **176:**3433-3447.
6. **Johnson, G. R., R. K. Jain, and J. C. Spain.** 2000. Properties of the trihydroxytoluene oxygenase from *Burkholderia cepacia* R34; an extradiol dioxygenase from the 2,4-dinitrotoluene pathway. Arch. Microbiol. **173:**86-90.
7. **Johnson, G. R., B. F. Smets, and J. C. Spain.** 2001. Oxidative transformation of amindinitrotoluene isomers by multicomponent dioxygenases. Appl. Environ. Microbiol. **67:**5460-5466.

8. **Johnson, G. R., and J. C. Spain.** 2003. Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. Appl. Microbiol. Biotechnol. **62:**110-123.
9. **Lessner, D. J., G. R. Johnson, R. E. Parales, J. C. Spain, and D. T. Gibson.** 2002. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. **68:**634-641.
10. **Nishino, S. F., G. Paoli, and J. C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66:**2139-2147.
11. **Nishino, S. F., J. C. Spain, and Z. He.** 2000. Strategies for aerobic degradation of nitroaromatic compounds by bacteria: process discovery to field application, p. 7-62. *In* J. C. Spain, J. B. Hughes, and H.-J. Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton Florida.
12. **Pridmore, R. D.** 1987. New and versatile cloning vectors with a kanamycin-resistance marker. Gene **56:**309-312.
13. **Ramos, J. L., M. M. Gonzalez-Perez, A. Caballero, and P. v. Dillewijn.** 2005. Bioremediation of polynitrated aromatic compounds: plants and microbes put up a fight. Current Opinion in Biotechnology **16:**275-281.
14. **Schmid, A., I. Vereyken, M. Held, and B. Witholt.** 2001. Preparative regio- and chemoselective functionalization of hydrocarbons catalyzed by cell free preparations of 2-hydroxybiphenyl 3-monooxygenase. J Mol Catal B: Enzym **11:**455-462.
15. **Smith, D. F., T. E. Kleindienst, and E. E. Hudgens.** 1989. Improved high-performance liquid chromatographic method for artifact-free measurements of aldehydes in the presence of ozone using 2,4-dinitrophenylhydrazine. J Chromatogr **483:**431-436.
16. **Spanggord, R. J., J. C. Spain, S. F. Nishino, and K. E. Mortelmans.** 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **57:**3200-3205.
17. **Suen, W.-C., B. E. Haigler, and J. C. Spain.** 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J. Bacteriol. **178:**4926-4934.

IX. Examination of 2,4-DNT degrading abilities through the use of genetically engineered bacterial strains

Summary

Genes encoding the 2,4-DNT degradative pathway were removed from environmentally isolated bacterial strains and integrated into strains commonly used in molecular genetics. The resulting genetically engineered constructs allow us to efficiently measure the properties of encoded enzymes within stable genotypes and under controlled conditions. The original strains, originating from contaminated soil and groundwater samples, are difficult to analyze using molecular genetic techniques. In the new constructs, genes can now be manipulated in strains grown under common laboratory conditions. They also relieve the dependence on media containing nitrotoluene pollutants for selection and maintenance of 2,4-DNT degradation.

Background

Genetic and molecular studies of the nitrotoluene degradation have shown that these catabolic pathways have undergone recent and continuing evolution [1, this study]. It is proposed that catalytic activity, regulation and expression of genes, and links between metabolic processes within strains utilizing nitrotoluenes are not fully developed in comparison to metabolic pathways of more natural compounds [2-4]. For example, strains growing on 2,4- and 2,6-DNT will accumulate considerable amounts of nitrate and metabolites, such as methylnitrocatechol, during growth [5-7]. Non-specific reduction of nitro groups within the cell generates toxic intermediates and dead end metabolites [8]. Catabolic enzymes are inhibited at very low concentrations of reaction products [9]. These and other inefficiencies are seen as bottlenecks in the bioremediation of sites contaminated with explosives and other nitroaromatic compounds [10]. Methods that allow us to measure and manipulate encoded activity of genes under controlled conditions are essential for developing degradative pathways to overcome these obstacles.

Nitroaromatic degradative pathways have been characterized in strains isolated only recently from contaminated soil and water samples [4, 7, 11-14]. In most cases, these strains have few, if any, established methods for genetic manipulation. Strains must be grown on nitroaromatic pollutants in order to maintain their genetic background and phenotype. These properties make it very difficult to analyze catalytic activity of the structural genes while expressed within their native hosts. Localizing the catabolic genes within common laboratory strains will allow us to easily study their inherent properties. We can then focus on and directly manipulate encoded catalytic properties utilizing well-established methods in molecular biology.

Results and Discussion

Genes encoding the 2,4-DNT degradative pathway were isolated from 2,4-DNT-degrading strains *Burkholderia* sp. strains DNT [7] and JS872 [6], and integrated into a hybrid operon for recombinant expression in laboratory strains (Figure IX-1). Genes encoding the initial catabolic step, *dntAαAbAcAd* were isolated from strain DNT, as described elsewhere [15]. The remaining

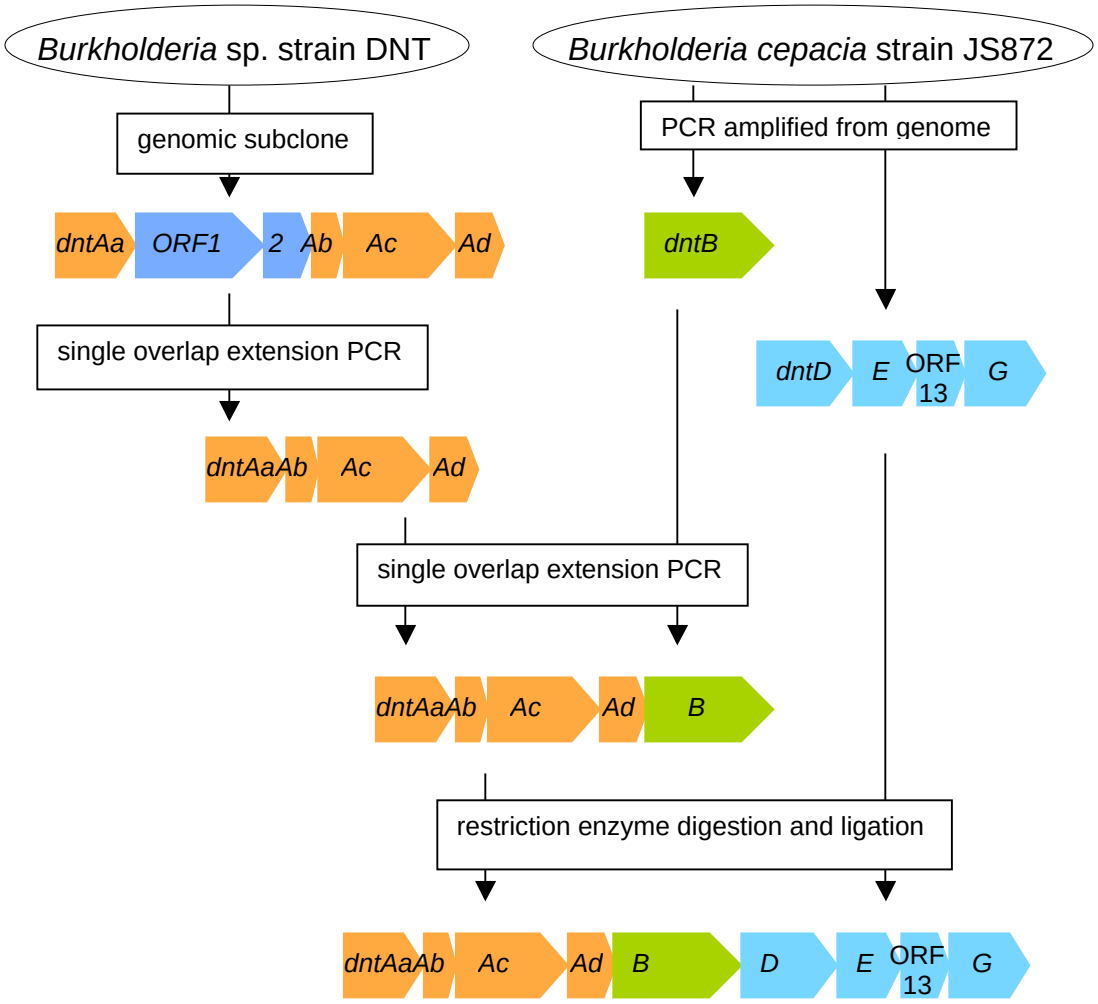


Figure IX-1. Schematic of engineered laboratory strains expressing *dnt* genes. Genes encoding the initial step of the 2,4-DNT degradative pathway was isolated on a genomic subclone from strain DNT. The remaining genes were PCR amplified from the genome of strain JS872 as two products, *dntB* and *dntDE*ORF13*G*. ORF1 and ORF2 were removed from in between *dntAa* and *dntAb* by single overlap extension (SOE) PCR [16]. The gene, *dntB* was attached to the end of the *dntA* gene also using SOE PCR. The remaining genes (*dntDE*ORF13*G*) were integrated with *dntAaAbAcAdB* using standard molecular genetic methods of restriction endonuclease digestion and enzymatic ligation. The complete *dnt* transcript was then incorporated with the *Pseudomonas*/*E. coli* expression vector, pMEKm12 [17] and transformed in *P. fluorescens* and *E. coli*.

genes encoding the 2,4-DNT degradative pathway were PCR amplified using boiled cells of strain JS872 as template. Primers were designed based upon sequences of *dnt* genes from *Burkholderia cepacia* strain R34 [1]. Genes were discovered within two amplicons: one containing *dntB* and another containing *dntD*, *dntE*, ORF13, and *dntG*. This arrangement of genes is similar to those isolated from strain DNT and R34 [1, 15]. Using single overlap extension PCR [16] and standard molecular genetic methods of restriction endonuclease digestion and enzyme-mediated ligation, the catabolic *dnt* genes were organized into one

transcript and placed under the control of *Pseudomonas*/*E. coli* expression vector pMEKm12 [17]. The new plasmid carrying the hybrid operon *dntAaAbAcAdBDE*ORF13*G* was used to transform *Escherichia coli* strain TOP10F' (Invitrogen, Carlsbad, CA) and *Pseudomonas fluorescens* (ATCC, Manasses, VA). Activities of the encoded enzymes were assayed during growth in rich medium containing appropriate antibiotics to maintain the replicating plasmid. Genes were induced when the cells were most active. Activity was either measured during growth or in resting cell suspensions. Nitrite release was determined as previously described [18].

When 2,4-DNT and 4M5NC were added to growing strains of *E. coli* and *P. fluorescens* expressing the *dnt* genes, nitrite was released, demonstrating that 2,4-DNT is degraded through the first two detoxification steps (Table IX-1 and Figure IX-2). When 0.2 mM 2,4-DNT was added to the *P. fluorescens* cultures, 0.4 mM of nitrite was released in approximately 2.5 hours, confirming that both nitro groups are removed from 2,4-DNT. Stochiometric amounts of nitrite (0.05 mM) also accumulated when 0.05 mM of 4M5NC was added to *P. fluorescens* cultures. Actively growing *E. coli* expressing *dnt* genes followed similar trends, except complete conversion of substrate was inhibited when 4M5NC was added to cultures. Only 0.038 mM nitrite was released from 0.05 mM 4M5NC before activity was lost.

To determine whether the ring fission dioxygenase was active in recombinant strains, 4-methylcatechol (4MC) was used as substrate, due to the limited availability and instability of the native substrate, 2,4,5-trihydroxytoluene [19]. In *E. coli* and *P. fluorescens* engineered strains, the yellow ring fission product from 4MC transformation was detected in active cultures, confirming active expression of *dntD* (Table IX-1). Furthermore, *P. fluorescens* was able to grow on solid media containing 2,4-DNT as the sole carbon source, albeit at a significantly slower rate than wild-type 2,4-DNT degrading strains (data not shown).

Conclusions

The structural *dnt* genes were isolated from two 2,4-DNT degrading strains and successfully expressed as active enzymes in *E. coli* and *P. fluorescens*. These two strains were chosen as hosts, due to their utility in molecular biology and easy genetic manipulation systems. The genes encoding the initial catalytic step of the 2,4-DNT pathway were specifically chosen from strain DNT, because their encoded activity attacks an amino-substituted 2,4-DNT substrate analog more efficiently than the other isolated 2,4-DNT dioxygenases. The hybrid operon created in this study will be used in metabolic engineering of a pathway for TNT degradation, which is proposed to generate amino-substituted DNT metabolites through reduction of one nitro group of TNT. The remaining *dnt* genes in the hybrid operon were chosen from strain JS872, due to the strain's considerably faster growth rate in comparison to other 2,4-DNT degraders, such as strain DNT and R34 [4].

2,4-DNT and 4M5NC are efficiently transformed by actively growing *P. fluorescens.* Because *P. fluorescens* is able to degrade naturally-occurring aromatic compounds, such as toluene, we expected the *dnt* genes would be quite functional in this strain. This engineered strain will grow on 2,4-DNT, but forms barely detectable pinpoint colonies on solid medium. The strain will not grow in liquid media with 2,4-DNT. We believe that growth on 2,4-DNT

occurs through a combination of several metabolic mechanisms in addition to the encoded activities of the structural genes. The very slow and no growth of *P. fluorescens* and *E. coli*, respectively, suggest that these strains are missing important components or do not induce them for efficient degradation of 2,4-DNT. Nitrite assimilation, substrate transport, and precise levels of gene dosage, governed by regulation mechanisms specific to native strains, are presumably essential to efficient growth and are not optimized in the engineered strains. As these additional characteristic are resolved, they can be incorporated into the engineered strains for controlled analysis.

Our novel recombinant strains will allow us to study specific mechanisms of enzyme activity, expression, regulation, and transport for efficient destruction of environmental pollutants. In their present genotype, the strains will be used to manipulate encoded substrate specificity and catalytic activity of the structural genes. As new insights into other elements of metabolism are identified, they will be integrated into the engineered strains for analysis. For example, regulatory genes and genetic sequences needed to induce native expression of genes will be incorporated into the recombinant strains and measured under controlled conditions. Our aim is to understand the conditions needed for allow the degradative pathway to operate at maximum efficiency, so we can implement them into a strategy to accelerate remediation of environmental contamination.

A recombinant expression system analogous to the one described above will be developed for the 2,6-DNT pathway. Our studies suggest that the 2,6-DNT degradative genes are not optimized within wild-type strains. The 2,6-DNT degradative genes in strain JS180 do not appear to be in close proximity with each other in the genome, nor are they controlled by normal mechanisms of regulation and expression. Integrating the genes within known genetic backgrounds and under controlled expression systems would be advantageous to their study. Furthermore, combining 2,4- and 2,6-DNT pathways within one genome will help us develop strains that degrade both isomers efficiently.

Table IX-1. Properties of *E. coli* and *P. fluorescens* expressing *dntAaAbAcAdBDE*ORF13*G*. Activity assays and nitrite determination were completed as described in text. Growth was determined on solid minimal medium containing 3 mM 2,4-DNT. Stochiometry between nitrite released and substrate consumed (μmoles) is listed in brackets. 4-methylcatechol (4MC) was used as the substrate to identify DntD activity, due to the instability of the native ring fission substrate, 2,4,5-trihydroxytoluene. Ring fission of 4MC by DntD was determined by spectrophotometric detection of a yellow ring fission product.

strain	NO_2^- release after addition of substrate: 2,4-DNT	(NO_2^- / 2,4-DNT)	4M5NC	(NO_2^- / 4M5NC)	conversion of 4MC	growth on 2,4-DNT
E. coli	+	(2.1)	+	(1:1)	+	-
P. fluorescens	+	(2.1)	+	(1:0.75)	+	+

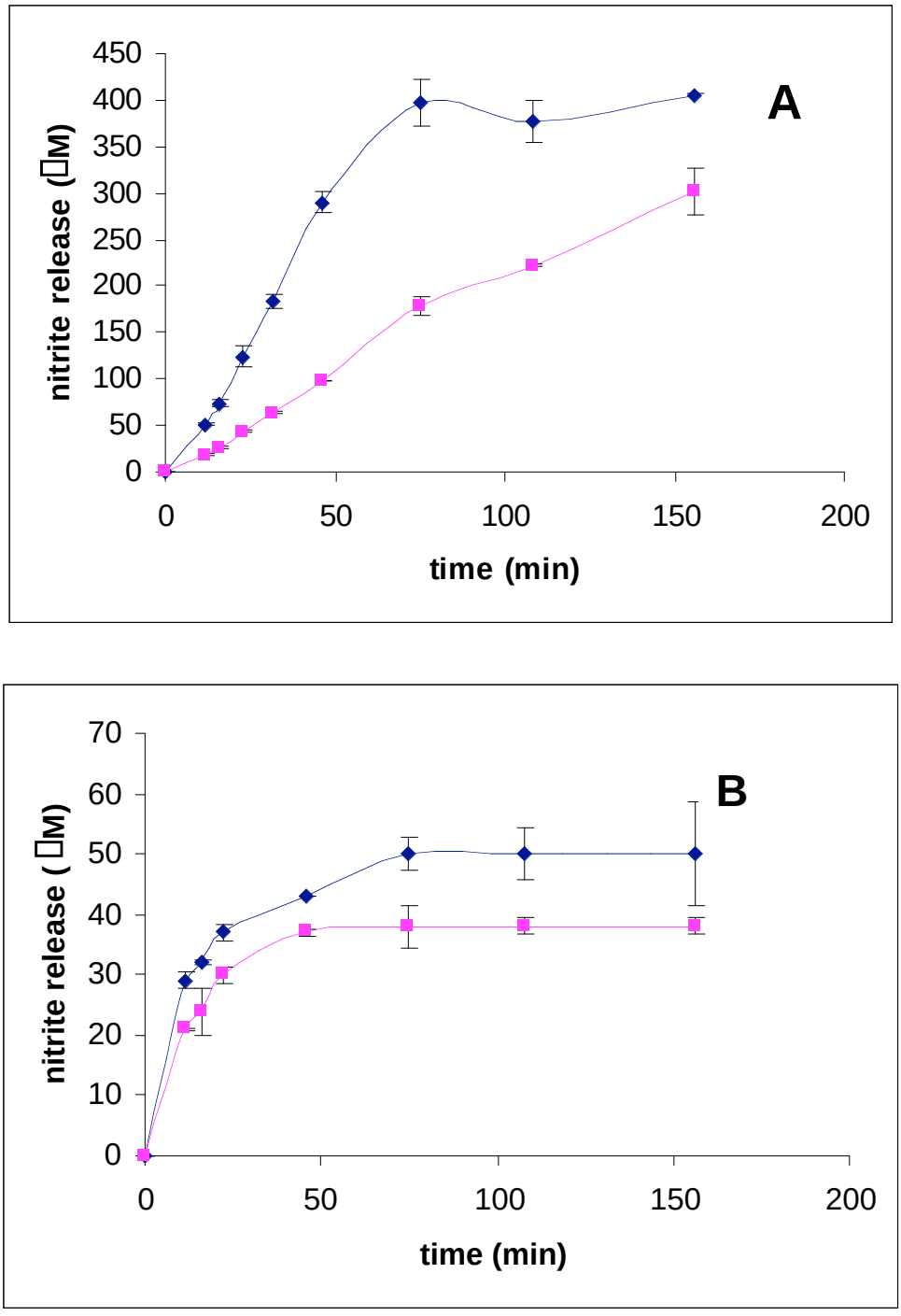


Figure IX-2. Conversion of 2,4-DNT and 4M5NC by *P. fluorescens* and *E. coli* expressing the hybrid *dnt* operon. Graphs show nitrite accumulation during transformation of 0.2 mM 2,4-DNT (A) and 0.05 mM 4M5NC (B) in active growing cultures expressing *dnt* genes. Cultures of *P. fluorescens* and *E. coli* are represented by the blue and magenta lines, respectively. Nitrite concentrations were determined using methods described in [18].

Literature Cited

1. **Johnson, G.R., R.K. Jain, and J.C. Spain.** 2002. Origins of the 2,4-dinitrotoluene pathway. J.Bacteriol. **184**:4219-4232.
2. **Lessner, D.J., et al.** 2003. Expression of the nitroarene dioxygenase genes in *Comamonas sp*. strain JS765 and *Acidovorax sp*. strain JS42 is induced by multiple aromatic compounds. J Bacteriol. **185**:3895-904.
3. **Johnson, G.R. and J.C. Spain.** 2003. Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. Appl. Microbiol. Biotechnol. **62**:110-123.
4. **Nishino, S.F., G. Paoli, and J.C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66**:2139-2147.
5. **Haigler, B.H., S.F. Nishino, and J.C. Spain.** 1994. Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. J.Bacteriol. **176**:3433-3447.
6. **Nishino, S.F., et al.** 1999. Mineralization of 2,4- and 2,6-dinitrotoluene in soil slurries. Environ. Sci. Technol. **33**:1060-1064.
7. **Spanggord, R.J., et al.** 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **57**:3200-3205.
8. **Spain, J.C., J.B. Hughes, and H.-J. Knackmuss,** eds. 2000. Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers: Boca Raton.
9. **Haigler, B.E., W.-C. Suen, and J.C. Spain.** 1996. Purification and sequence analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp. strain DNT. J.Bacteriol. **178**:6019-6024.
10. **Nishino, S.F. and J.C. Spain,** 2001. Identification of bottlenecks to the in situ bioremediation of dinitrotoluene, in Bioremediation of energetics, phenolics, and polycyclic aromatic hydrocarbons: the sixth international in situ and on-site biormediation symposium, V.S. Magar, et al., Eds. Battelle Press: San Diego. p. 59-66.
11. **Nishino, S.F. and J.C. Spain .** 1993. Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes.* Appl. Environ. Microbiol. **59**:2520-2525.
12. **Haigler, B.E. and J.C. Spain.** 1993. Biodegradation of 4-nitrotoluene by *Pseudomonas* sp. strain 4NT. Appl. Environ. Microbiol. **59**:2239-2243.
13. **Haigler, B.E., W.H. Wallace, and J.C. Spain.** 1994. Biodegradation of 2-nitrotoluene by *Pseudomonas sp.* Strain JS42. Appl. Environ. Microbiol. **60**:3466-3469.
14. **Haigler, B.E. and J.C. Spain.** 1991. Biotransformation of nitrobenzene by bacteria containing toluene degradative pathways. Appl. Environ. Microbiol. **57**:3156-3162.
15. **Suen, W.-C. and J.C. Spain.** 1993. Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation. J.Bacteriol. **175**:1831-1837.
16. **Shuldiner, A.R., et al.** 1991. Ligase-free subcloning: a versatile method to subclone polymerase chain reaction (PCR) products in a single day. Anal. Biochem. **194**:9-15.
17. **Lu, S.E., B.K. Scholz-Schroeder, and D.C. Gross.** 2002. Construction of pMEKm12, an expression vector for protein production in *Pseudomonas syringae.* FEMS Microbiol. Lett. **210**:115-21.
18. **An, D., D.T. Gibson, and J.C. Spain.** 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. J.Bacteriol. **176**:7462-7467.

19. **Johnson, G.R., R.K. Jain, and J.C. Spain.** 2000. Properties of the trihydroxytoluene oxygenase from *Burkholderia cepacia* R34; an extradiol dioxygenase from the 2,4-dinitrotoluene pathway. Archives of Microbiology. **173**:86-90.

Final Conclusions and Future Outlook

The goal of this project was to develop a strategy and biocatalyst for the aerobic degradation of TNT supported by DNT as the electron donor. The results described here have made substantial progress towards that end. We have:

1. Discovered enzymes in DNT degrading bacteria that catalyze all of the individual steps in the conversion of TNT to ring-fission products that contain no nitro groups and are likely to be biodegraded by soil bacteria.
2. Demonstrated that bacteria degrading DNT can cometabolize TNT.
3. Elucidated the origin of nitroarene dioxygenases.
4. Characterized substrate specificities of nitrorarene dioxygenases.
5. Elucidated the regulation of nitroarene dioxygenases and identified a potential regulatory gene for a constructed TNT degrading strain.
6. Cloned and sequenced genes for key enzymes in the 2,6-DNT degradation pathway.
7. Discovered nitroreductases specific for nitro groups at the 2-carbon position of TNT and characterized nitroreductase activities in potential host strains.
8. Demonstrated that multiple operons for 2,4-DNT degradation can be integrated as a single unit and put under the control of a single regulator capable of heterologous expression.

The studies described herein advanced the understanding of the system to the point that it is possible to envision an ideal biocatalyst and detail the steps necessary to develop such a microbe. The organism would contain a constitutive nitroreductase that would specifically attack the nitro group of TNT at the 2-carbon position to produce 2ADNT. As the work reported above has demonstrated, 2ADNT and subsequent metabolic intermediates are subject to catalysis by enzymes of the 2,4-DNT-degradation pathway. Although reduction of the nitro group at the 4-carbon position is also a possibility, the resulting amino compound would be a 2,6-DNT analog. Enzymes of the 2,6-DNT-degradation pathway can catalyze further transformations of 4ADNT, but because the 2,6-DNT-degradation pathway is less understood and less robust than the 2,4-DNT pathway, it makes sense to work with the 2,4-DNT system. Directed evolution would improve the catalytic activity of each enzyme in the pathway towards the TNT metabolites. Higher activities or enhanced coordination of successive transformation steps would help to avoid misrouting of the intermediates towards dead end products.

The activator protein of the 2NT degradation pathway is a good candidate to regulate the expression of the proposed pathway. NtdR causes a high basal level of expression even in the absence of inducers, but it results in expression of the nitroarene dioxygenases genes at even higher levels when mono-, di- and aminodinitrotoluenes are present. This discovery presents the possibility of expressing the initial dioxygenase in the absence of 2,4-DNT with just ADNT as the inducer. The 2,4-DNT degradation pathway is organized in multiple operons in the 3 strains that have been analyzed in detail. We do not yet know how the operons encoding the lower pathways are regulated, but we can bypass that difficulty if we integrate the structural genes for DNT-degradation into a single hybrid operon under the control of NtdR. If one of the native 2,4-DNT-degrading strains were used as the host for the hybrid operon, we might readily overcome

the difficulties of efficient expression of the hybrid operon that were observed with *P. fluorescens* and *E. coli.*

B. cepacia JS872, or another native DNT-degrading strain in our collection, may be a candidate host for the final engineered pathway. The attractiveness of strain JS872 lies in the fact that it contains a nitroreductase that is specific for the nitro group at the 2-carbon position. Whether the expression of the nitroreductase is or could be made constitutive is a tractable problem. Strain JS872 also already contains the necessary metabolic background for efficient expression of the 2,4-DNT degradation pathway, so only minimal changes, if any, to the host background for expression of the hybrid operon are likely to be required. If a *Burkholderia* presents regulatory problems because it is a potential pathogen, then other strains that also degrade 2,4-DNT well, such as one of the *Achromobacter xylosoxidans* isolates might be an appropriate host.

The goal of the project was to develop strategies for DNT-dependent cometabolism of TNT. Therefore, sites where DNT and TNT contamination coexist should be considered for treatment with the aerobic cometabolic strategy. Such a strategy will clearly lead to mineralization of DNT and substantial, if not complete metabolism of TNT. The final products of the aerobic oxidation of TNT have yet to be determined. Based on our experiments they will be ring fission products containing no nitro groups (Figure O-2). They will be polar and water-soluble and can be expected to biodegrade in soil. They might bind to clays and the amino groups might lead to covalent binding with soil, but the predominant fate would be expected to be biodegradation. Future experiments with radiolabelled or ^{13}C TNT will be required to reveal the behavior of the metabolites. If fully realized, the engineered organism would be a true TNT-mineralizing bacterium and be free of the constraints now associated with cometabolic strategies for TNT degradation. It could be used for ground water remediation as well as soil applications. The lack of a requirement for primary growth substrates other than the contaminants of concern means that it could be used to effectively seed firing ranges and remote contaminated sites without the concern for addition of massive amounts of other compostable materials, thus avoiding both materials handling and storage costs as well as disruption of the primary use of the site.

To achieve this end, the following efforts need to be undertaken:

- Characterize a nitroreductase specific for the 2-carbon position.
- Make the nitroreductase expression constitutive if not already constitutive in the candidate host.
- Express the hybrid 2,4-DNT degradation operon in a host that is a native DNT-degrader under the regulation of *ntdR*.
- Determine the fate of ring fission products from TNT.
- Test the engineered organism for the ability to grow on TNT.
- Subject the engineered organism to rounds of directed evolution as necessary to improve TNT degradation.
- Test the final organism in microcosms or bioreactors.

Appendix

List of Technical Publications

Peer-reviewed articles

G.R. Johnson, J.C. Spain, and B.F. Smets. 2001. Oxidative transformation of amino-dinitrotoluene isomers by multicomponent dioxygenases. Appl. Environ. Microbiol. **67:**5460-5466.

D.J. Lessner, Glenn R. Johnson, R.E. Parales, J.C. Spain, and D.T. Gibson. 2002. Molecular characterization and substrate specificity analysis of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. **68:**634-641.

G.R. Johnson, R.K. Jain, and J.C. Spain. 2002. Origins of the 2,4-dinitrotoluene pathway. J. Bacteriol. **184:**4219-4232

R.E. Parales, N.C. Bruce, A. Schmid, and L.P. Wackett. 2002. Biodegradation, biotransformation, and biocatalysis (B3). Appl. Environ. Microbiol. **68:**4699-4709.

D.J. Lessner, R.E. Parales, S. Narayan and D.T. Gibson. 2003. Expression of the nitroarene dioxygenase genes in *Comamonas* sp. strain JS765 and *Acidovorax* sp. strain JS42 is induced by multiple aromatic compounds. J. Bacteriol. **185:**3895-3904.

J.D. Fortner, C. Zhang, J.C. Spain, and J.B. Hughes. 2002. Soil column evaluation of factors controlling biodegradation of DNT in the vadose zone. Environ. Sci.Technol. **37:**3382-3391.

G.R. Johnson and J.C. Spain. 2003. Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. Appl. Microbiol. Biotechnol. **62:**110-123.

K.-S. Lee, J.V. Parales, R. Friemann and R.E. Parales. 2005. Substrate specificity of 2-nitrotoluene dioxygenase: effect of specific amino acid substitutions at the active site of the enzyme. J. Ind. Microbiol. Biotechnol. **32:**465-473.

R.E. Parales, R. Huang, C.-L. Yu, J.V. Parales, F.K.N. Lee, M.M. Ivkovic-Jensen, W. Liu, D.J. Lessner, R. Friemann, S. Ramaswamy, and D.T. Gibson. 2005. Purification, characterization, and crystallization of the components of the nitrobenzene and 2-nitrotoluene dioxygenase enzyme systems. Appl. Environ. Microbiol. **71**:3806-3814.

R. Friemann, M.M. Ivkovic-Jensen, D.J. Lessner, C.-L. Yu, D.T. Gibson, R.E. Parales, H. Eklund and S. Ramaswamy. 2005. Structural insights into the dioxygenation of nitroarene compounds: The crystal structure of the nitrobenzene dioxygenase. J. Mol. Biol. **348**:1139-1151.

K.-S. Ju and R.E. Parales. Control of substrate specificity by active site residues in nitrobenzene dioxygenase. Appl. Environ. Microbiol. (Submitted).

Book Chapters and Reviews

C.H. Ward, J.B. Hughes, G.A. Pope, M. Delshad, V. Dwaranath, J. Spain, S. Nishino, J.S. Fruchter, V.R. Vermeul, M.D. Williams, and J.E. Szecsody. 2002. In situ treatment technologies. In: D. Reible and K. Demnerova (eds.) Innovative Approaches to the On-Site Assessement and Remediation of Contaminated Sites. Kluwer Acacemic Publishers, Dordrecht, The Netherlands.

R.E. Parales and S.M. Resnick. 2004. Aromatic hydrocarbon dioxygenases. In: Soil Biology, vol. 2, Biodegradation and Bioremediation, A. Singh and O.P. Ward, (eds.). pp. 175-195. Springer-Verlag, Germany.

R.E. Parales and J.D. Haddock. 2004. Biocatalytic degradation of pollutants. Curr. Opin. Biotechnol. **15:** 374-379.

S.F Nishino and J.C. Spain. 2004. Catabolism of nitroaromatic compounds. *In* J.-L. Ramos (ed.), The Pseudomonads Vol III. Biosynthesis of macromolecules and molecular metabolism. pp. 575-608. Kluwer Academic/Plenum Publishers

R.E. Parales and J.L. Ditty. 2005. Laboratory evolution of catabolic enzymes and pathways. Curr. Opin. Biotechnol. **16:** 315-325.

R.E. Parales and S.M. Resnick. 2005. Ring hydroxylating dioxygenases. In: *Pseudomonas* Volume 4: Molecular Biology of Emerging Issues, J.-L. Ramos and R.C. Levesque, (eds.). Kluwer Academic/ Plenum Publishers, New York. (In press).

R.E. Parales and S.M. Resnick. 2006. Applications of aromatic hydrocarbon dioxygenases. In: Biocatalysis in Biotechnological and Pharmaceutical Industries, R.N. Patel, (ed.) CRC Press, Boca Raton, Fla. (In press).

Published Technical Abstracts

D.J. Lessner, R.E. Parales, and D.T. Gibson. "Regulation of the genes encoding nitrobenzene and 2-nitrotoluene dioxygenases." Abstr. Biodegradation, Biotransformation, and Biocatalysis (B3). San Juan, Puerto Rico, October 2001.

J.C. Spain. "Biodegradation Pathways as Sources of Novel Biocatalysts." Abstr. Biodegradation, Biotransformation, Biocatalysis (B3). San Juan, Puerto Rico, October 2001.

J.V. Parales, R.E. Parales, S. Wolfe, and D.T. Gibson. "Cloning and characterization of Rieske non-heme iron oxygenase genes from isolates that degrade nitroaromatic compounds". Abstr. 10th Biocatalysis and Bioprocessing Conference, Iowa City, IA, October 2001.

R.E. Parales, D.J. Lessner, J.C. Spain, and D.T. Gibson. "Regulation of the nitroarene dioxygenase genes in bacterial strains that degrade nitrobenzene and 2-nitrotoluene." Abstr. Partners in Environmental Technology Technical Symposium and Workshop. Washington, D.C., May 2001.

D.J. Lessner, R.E. Parales, and D.T. Gibson. "Expression of nitroarene dioxygenase genes is induced by multiple aromatic compounds." Abstr. Q-238,102nd General Meeting of the American Society for Microbiology, Salt Lake City, Utah, May 2002.

J.V. Parales, K.-P. Choi, R.E. Parales, and D.T. Gibson. "Cloning and characterization of Rieske non-heme iron oxygenase genes from 2,6-dinitrotoluene degrading bacteria." Abstr. Q-231, 102nd General Meeting of the American Society for Microbiology, Salt Lake City, Utah, May 2002.

D.J. Lessner, R.E. Parales, and D.T. Gibson. "Promiscuous expression of nitroaromatic dioxygenase genes? Induction by multiple aromatic compounds." Abstr. Annual Meeting of the Society for Industrial Microbiology, Philadelphia, PA., August 2002.

D.J. Lessner, R.E. Parales, and D.T. Gibson. "The involvement of LysR-type transcriptional regulators in the expression of nitroarene dioxygenases." Abstr. 11th Biocatalysis and Bioprocessing Conference, Iowa City, IA, October 2002.

C.A. Kachel, R. Giannone, R.E. Parales, G.R. Johnson, J.C. Spain, and G.J. Zylstra. "Nucleotide sequence analysis of the *Burkholderia* sp. strain DNT large catabolic plasmid for dinitrotoluene degradation." Abstr. 103rd General Meeting of the American Society for Microbiology, Washington, D.C., May 2003.

T.J. Fleischmann, S.L. Ivey, K.C. Walker, J.C. Spain, and A.M. Craig. 2003. Anaerobic transformation of 2,4,6-TNT by bovine ruminal microbes. ." Abstr. 103rd General Meeting Meeting of the American Society for Microbiology, Washington, D.C., May 2003.

R.E. Parales. "Evolution of Nitrobenzene and 2-Nitrotoluene Degradation Pathways." Abstr. Army Research Office Biosciences Workshop, Cashiers, NC, April 2004.

G.R. Johnson and J.C. Spain. “Synthesis of novel catechols using bacterial nitroarene dioxygenases”. Abstr. 104rd General Meeting Meeting of the American Society for Microbiology, New Orleans, LA, May 2004.

K.-S. Ju and R.E. Parales. "Bacterial nitroarene dioxygenases: Identification of active site residues involved in regioselectivity and enantioselectivity." Abstr. 39th Western Regional Meeting of the American Chemical Society. Sacramento, CA, October 2004.

J.V. Parales, K.S. Lee, and R.E. Parales. "Probing the active site of 2-nitrotoluene 2,3-dioxygenase". Abstr. 13th Biocatalysis and Bioprocessing Conference, Iowa City, IA, October 2004.

D.M. Eby and J.C. Spain. “Ring fission of 3-methyl-4-nitrocatechol during 2,6-dinitrotoluene degradation is catalyzed by a novel extradiol dioxygenase”. Abstr. 105rd General Meeting of the American Society for Microbiology, Atlanta, GA. June 2005.

G.R. Johnson, D.M. Eby, and J.C. Spain. “Transformation of aminomethylnitrocatechol isomers - towards an engineered pathway for aerobic 2,4,6-trinitrotoluene degradation”. Abstr. 105rd General Meeting of the American Society for Microbiology, Atlanta, GA. June 2005.

K.-S. Ju, R. Friemann, S. Ramaswamy, and R.E. Parales. "Defining amino acids that control regiospecificity in nitrobenzene 1,2-dioxygenase." Abstr. 105rd General Meeting of the American Society for Microbiology, Atlanta, GA. June 2005.

R.E. Parales. "Evolution of Bacterial Pathways for Nitroarene Degradation." Abst. XIth International Congress of Bacteriology and Applied Microbiology, San Francisco, CA. July 2005.